EXCITATORY SIGNAL TRANSDUCTION MEDIATED BY INOSITOL PHOSPHOLIPID METABOLISM IN LOBSTER *(PANULIRUS ARGUS)* OLFACTORY RECEPTOR NEURONS

By

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1993

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by

Debra Ann Fadool

TO MY THREE MEN

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By

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Major Department: Zoology

Appropriate primary sustained culture conditions were developed to study signal transduction in *Panulirus argus* olfactory receptor neurons (ORNs). Neurons were cultured in a modified Liebowitz media supplemented with salts, vitamins, L-glutamine, low dextrose, and either fetal calf serum or lobster haemolymph. The nature of the adequate stimuli, the degree of tuning of the cells, the threshold of sensitivity, and the dual polarity of the odor-evoked currents were consistent with chemosensitivity in the cultured ORNs being olfactory.

The magnitude of the odor-evoked currents was significantly increased or decreased by nonhydrolyzable analogs of GTP and GDP, respectively, and not perturbed by pertussis and cholera toxins, implying that a class of bacterial toxin-insensitive G-proteins was mediating signal

transduction. An antibody directed against $G_{o\alpha}$ immunolabelled a 40.5 kDa band in an enriched membrane preparation of ORN outer dendrites and, along with an antibody directed against $G_{q\alpha}$, selectively decreased the odor-evoked inward current within 10 min of initial perfusion.

Inositol 1,4,5-trisphosphate (IP_3) selectively evoked an inward current in the ORNs. Application of IP_3 to the inside face of cell-free patches of ORN plasma membrane directly gated two ion channels that differed in conductance, voltage dependence, and dwell-time kinetics. An antibody directed against an intracellular, cerebellar IP_3 receptor recognized a protein of similar molecular weight to the mammalian receptor in the ORNs and was found to increase selectively the odor-evoked inward currents and IP_3-activated unitary currents in the lobster ORNs.

Modulation of channel gating or ion permeation was observed in both IP_3-gated channels in response to elevated pH or $[Ca^{2+}]_i$. Both channels mimicked the pharmacology of the macroscopic odor-evoked inward currents. Ion substitution suggested that the small-conductance channels were nonselective for cations and that the large-conductance channels were either nonselective between Na^+ and Ca^{2+} or were selective for Ca^{2+}.

The direct metabolite of IP_3, inositol 1,3,4,5-tetrakisphosphate (IP_4), gated an ion channel that differed in conductance, density, kinetics, and voltage sensitivity from those activated by IP_3. The IP_4-gated channel mutually interacted with IP_3-gated channels to alter the open probability of the channels.

CHAPTER 1
INTRODUCTION

Amino Acid Receptors and Aquatic Chemoreception

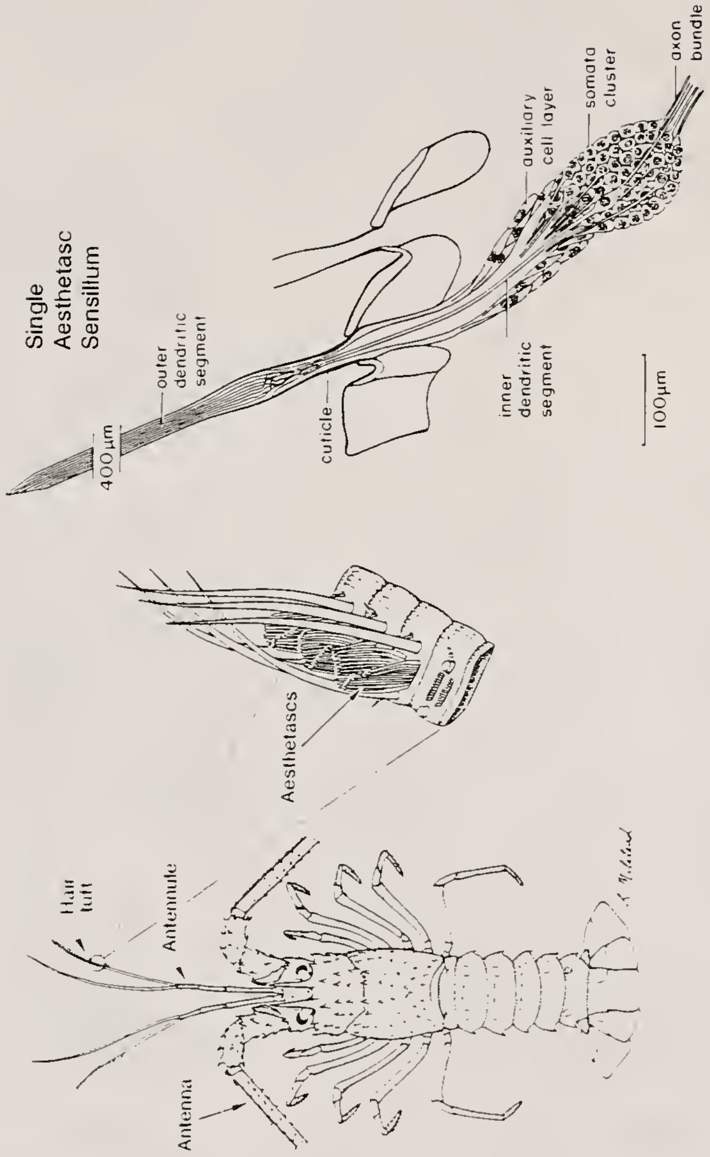
The past decade has shown an explosive interest in excitatory amino acids (EAAs) as important neurotransmitters of the mammalian central nervous system (CNS). Research efforts have elucidated the synaptic role of EAAs, the EAA subclasses of receptors through pharmacological techniques, the mode of signal transduction, and the extent to which EAAs are involved in brain function. To date, EAAs are implicated in a wide range of physiological phenomena including the processing of sensory information, cognitive processes, learning, and memory. Dysfunction of receptor-ligand interaction and subsequent transduction can lead to neurological disorders such as epilepsy, spasticity, neurodegeneration after a stroke, as well as Huntington's and Alzheimer's disease (Croucher et al., 1984; Watkins et al., 1990). The development of selective agonists and antagonists for distinct EAAs receptors has implicated amino acids in synaptic transmission throughout various regions of the CNS/brain and has offered pharmacological intervention in treating many of these human neuronal diseases (Lodge and Collingridge, 1990).

Amino acid receptors are not limited to the mammalian CNS. Feeding stimulants for many aquatic organisms, both fish and invertebrates, are simple hydrophilic tissue metabolites, mainly quaternary ammonium compounds, nucleotides, amines, and amino acids (e.g., Ache and Carr, 1990). Chemical analysis found 19 of the 20 common amino acids in muscle tissue of the blue crab, a typical prey organism for the lobster (Carr et al., 1984). Consequently, aquatic organisms typically possess well defined chemosensory structures containing primary chemosensory receptor neurons that are responsive to micromolar to picomolar amounts of amino acids and related molecules. Unlike the mammalian brain, where amino acid receptors are buried and widely distributed in tissue, these "external" amino acid receptors in aquatic organisms are readily accessible and often highly enriched in the chemosensory structures.

Large decapod crustaceans such as crabs and lobsters have such a chemosensory structure on the lateral branch of the first antenna, commonly referred to as the "olfactory" organ or antennule. It consists of a tuft of cuticular sensilla. Each sensillum contains an average of 318 bipolar receptor neurons (Grünert and Ache, 1988), yielding upwards of 450,000 receptor neurons per antennule (Figure 1-1). Outer dendrites of each bipolar cell branch extensively, filling the distal 80% of the lumen of the sensillum.

Figure 1-1. (LEFT) Large decapod crustaceans such as crabs and lobsters have a chemosensory structure on the lateral branch of the first antenna, commonly referred to as the "olfactory" organ or antennule. (MIDDLE) The olfactory organ is divided into rings called annuli. Each annulus contains of a tuft of innervated hairlike cuticular structures called aesthetasc sensilla, which are arranged in a zig-zag like pattern surmised favorable for sampling and clearing of odorants (Gleeson et al., 1993). (RIGHT) Dendrites of each bipolar cell branch extensively, filling the distal 80% of the lumen of the sensillum. Odorants reach the dendrites, the presumed site of signal transduction, through a porous cuticle surrounding the sensilla. The somata of 318 bipolar receptor neurons are clustered at the base of each sensillum, yielding upwards of 450,000 receptor neurons per antennule. The axons from each cluster project, without synapsing, to the CNS.

Modified, with permission from Grünert and Ache, 1988.


Antenna
Hair tuft
Antennule
Aesthetascs
Single Aesthetasc Sensillum
outer dendritic segment
400µm
cuticle
inner dendritic segment
100µm
auxiliary cell layer
somata cluster
axon bundle

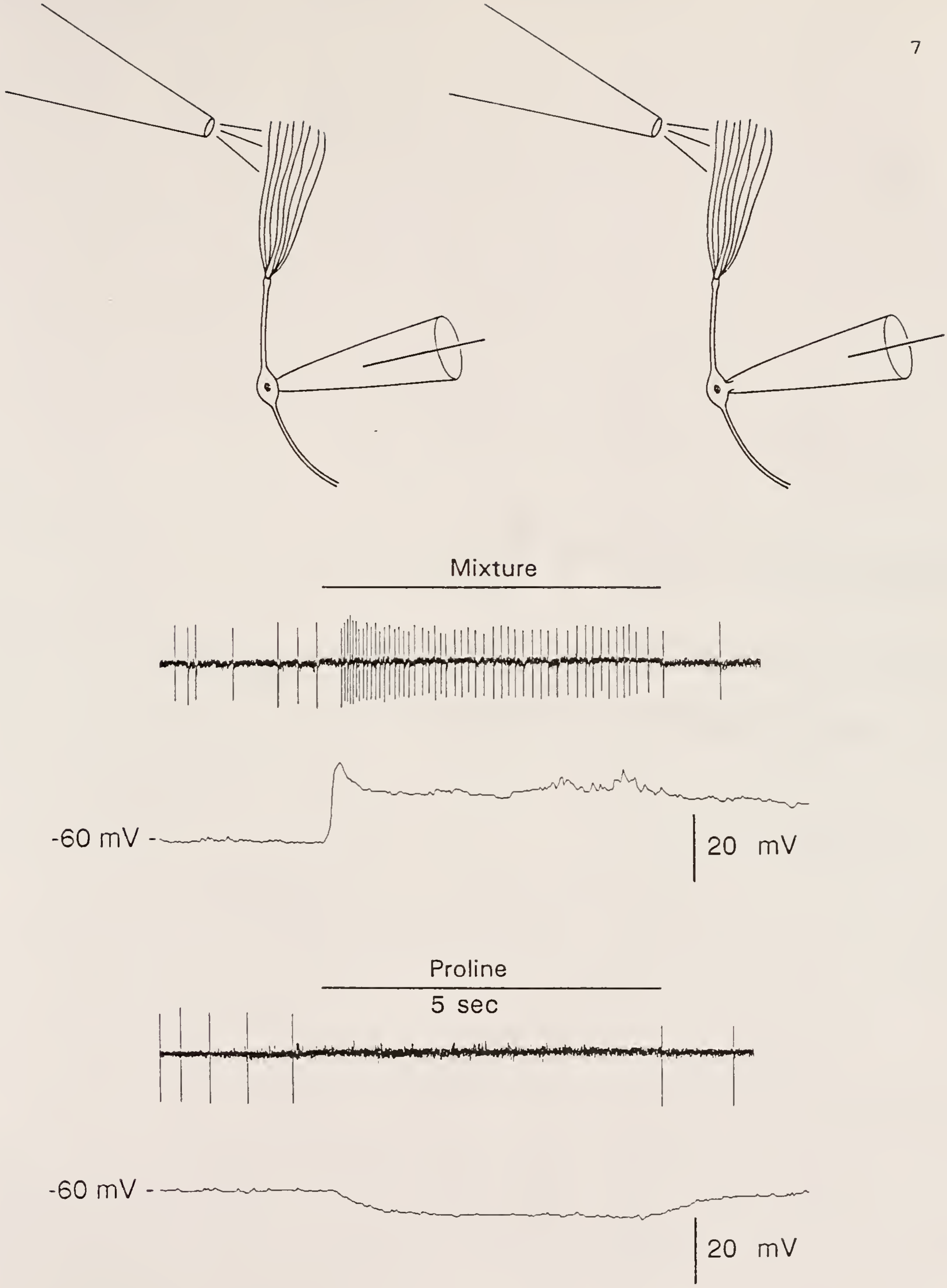
Odorants reach the dendrites through the porous cuticle surrounding the sensilla. The somata of the receptor neurons are clustered at the base of each sensillum and the axons from each cluster project, without synapsing, to the CNS.

Olfactory receptor neurons (ORNs) in lobsters have been the object of considerable research (review, Ache and Derby, 1985). The ORNs can be studied *in situ* (Ache et al., 1989) or harvested to produce a preparation of almost pure chemoreceptive neurons for tissue culture (Chapter 2), biochemistry (Chapter 4-5), or molecular biology (Trapido-Rosenthal et al., 1993; Munger and Ache, in press).

Of background relevance to the present project is that amino acids presented in mixtures (i.e., co-released) typically are less stimulatory to the neurons, as measured by intensity of the electrophysiological response of the neuron, than when presented individually and the responses summed. This phenomenon, called mixture suppression, is potentially the result of several underlying physiological mechanisms (Ache et al., 1988). Recently it was shown that amino acid "odorants" could inhibit as well as excite lobster olfactory neurons and, more importantly, that these two processes could occur in the same neuron (McClintock and Ache, 1989b; Michel et al., 1991; Figure 1-2). In other words, the excitatory action of one amino acid can be tempered by the inhibitory action of another (Michel et al.,

Figure 1-2. Amino acid "odorants" can inhibit as well as excite the lobster olfactory receptor neuron (ORN). Shown is a single ORN that was first recorded in the cell-attached configuration (top left diagram) to monitor action potential increase and decrease in frequency under stimulation by an excitatory odor mixture (top trace, upper recording) and an inhibitory odor (top trace, lower recording), respectively. The cell membrane was then ruptured to achieve the whole-cell recording configuration (top right diagram), in which the ORN responded with a membrane depolarization (bottom trace, upper recording) and then hyperpolarization (bottom trace, lower recording) under the same stimulation protocol. This singular experiment demonstrated that more than one transduction pathway for amino acids was present in these neurons.

Taken with permission from Michel et al., 1991.


Mixture
-60 mV -
20 mV
Proline
5 sec
-60 mV -
20 mV

1991; Michel and Ache, in press). This finding strongly suggested that more than one transduction pathway for amino acids is present in these neurons. What these pathways were remained unknown.

Molecular Mechanisms of Signal Transduction

Perhaps classically the most studied and hence best understood transduction pathway in neurons is (1) ligand binds to a receptor protein, (2) which induces the activation of an adenylate cyclase enzyme through G-protein coupling, (3) then the cyclic adenosine monophosphate (cAMP) end product acts directly or indirectly on an ion channel, (4) to induce a generator current. When I commenced my doctoral study, olfactory transduction, defined as the mechanism that converts a chemical signal (odor) into an electric signal (current) was not well perceived. It was known that a preparation enriched in olfactory cilia contained an odor-activated adenylate cyclase (Pace et al., 1985), suggesting that olfactory transduction had some resemblance to that typical of many neurons. Not all odorants had the capacity to evoke significant elevations in adenylate cyclase, suggesting cAMP-mediated transduction was not the sole mechanism. Nonetheless, a conductance directly gated by cyclic nucleotides (cAMP & cGMP) was localized to the olfactory cilia (Nakamura and Gold, 1987), which soon became established as the site of olfactory signal transduction (Kurahashi, 1989; Firestein et al., 1990).

Soon after, an olfactory specific G-protein, G_{olf}, was cloned and localized to the ciliary layer of rat ORNs (Jones and Reed, 1990). The fact that cholera toxin ribosylated proteins in the outer dendritic segments of lobster ORNs, implicated a GTP-dependent protein in at least one of the two transduction processes in these neurons (McClintock et al., 1990). Yet in lobster, complex odor mixtures or identified stimulatory odor molecules failed to alter levels of cAMP (McClintock et al., 1989). Hence, in lobster ORNs, data did not support cAMP as the candidate G-protein linked second messenger.

Several other molecular mechanisms had been implicated in signal transduction in other systems, providing me useful avenues of investigation at the commencement of my study. They included the following:

(1) Amino acids could directly gate channels, where the channel was contained within the receptor protein or where the channel and receptor were inherently two different proteins coupled directly or through a G-protein (Codina et al., 1987; Vogt et al., 1990).

(2) Amino acids could activate a guanylate cyclase to increase cGMP levels to directly activate a channel or indirectly activate a channel by phosphorylation via protein kinase C (PKC) (Vogt et al., 1990).

(3) Amino acids could operate through an inositol phosphate second messenger system (Berridge, 1989). In this scheme

stimulation of cell-surface amino acid receptors hydrolyzes a membrane bound inositol phospholipid, which produces two second messengers--neutral membrane bound diacylglyceral (DAG) and water soluble inositol 1,4,5-triphosphate (IP_3). DAG remains in the plane of the membrane (Nishizuka, 1984, 1988) to activate PKC which in turn could activate a channel. IP_3 diffuses into the cytosol releasing Ca^{2+} from internal stores, causing Ca^{2+} wave oscillations, and activating Ca^{2+} channels (Berridge and Irvine, 1989). IP_3 could additionally activate a channel directly (Kuno and Gardener, 1987). Inositol 1,3,4,5-tetrakisphosphate (IP_4), the direct metabolite of IP_3, may act as an additional messenger (Irvine, 1990).

The difficulty in studying olfactory transduction in *Panulirus argus* resided in the fact that the transduction machinery was hypothesized to lie in the outer dendritic membrane of the neuron, as was true for the analogous structure in vertebrates (above section; Nakamura and Gold, 1987; Kurahashi, 1989; Firestein et al., 1990). The thin diameter of this process (< 0.2 μm) made direct electrode seals virtually impossible. Recording from the neuron at the more accessible soma depended upon recording a transduction current from as far as 1000 μm away and often produced an inadequate voltage-clamp. I hypothesized that if the lobster ORNs could be sustained in primary cell culture without altering their voltage- or odor-evoked properties, then perhaps the cells would assume a form that was more amendable for voltage-clamping and for studying transduction. If lobster ORNs *in vitro* mimicked the electrical properties of their counterparts *in situ,* then the cultured cells would be an excellent model for studying the elements of olfactory signal transduction, particularly in a cell that was suspected of having multiple mechanisms.

Single-Channel Recording

"We should remember that our living systems are essentially watery saline systems that, through a variety of electrodes, are coupled to physical instruments" (Author unknown). This section provides an elementary background in electrophysiological techniques as a preface to the experimental results of Chapters 2-7. Electrophysiology is a powerful tool to probe the molecular events occurring at specialized structures along excitable membranes. The standard approach is to detect and measure bioelectrical potentials--yet by far the majority of bioelectrical potentials are so small that a sensitive apparatus is required to detect them. For crustacean olfactory receptor neurons (*Panulirus argus*) instruments which can detect a few to 100 pA are required. Electrode is a general term for a device that couples the biological preparation to electrical instrumentation. For recording from lobster ORNs, I fabricated electrodes from boralex silicate glass. These electrodes were fire polished to a final tip diameter of

1 μm to permit recordings from the small, 8-15 μm diameter cells. Good electrical contact must exist between electrode and cell. For an unknown reason first discovered by Ling and Gerard (1949), the glass of the electrode forms an excellent seal with the lipids in the nerve cell-membrane so that the current pulse, once converted to voltage by a headstage current-to-voltage converter, is received at an amplifier. The general function of an amplifier is to increase the voltage of a bioelectrical signal so that it can be displayed or further processed by a read-out device, such as a cathode ray oscilloscope (CRO), a strip chart recorder, or a computer.

On either side of an excitable membrane there are varying concentrations of ionic species. The primary species are Na^+, K^+, Ca^{2+}, and Cl^-. This electrical and chemical gradient, established by the semi-permeable nature of the cell membrane, is defined as the potential difference. Ionic flow across the cell membrane is confined to specialized protein pores called channels (Figure 1-3). Channels in lobster ORNs are "gated" or opened, as in other neurons, in response to one of two applied stimuli: (1) ligands such as odors, second messenger molecules, or neurotransmitters and (2) voltage as an electrical signal or stimuli. The movement of ions through these channels is essentially a flow of charge (or a current), which can be measured by a technique called voltage-clamping. In

Figure 1-3. (TOP) Ionic flow across the cell phospholipid bilayer is confined to specialized protein pores called channels that span the phospholipid bilayer. (MIDDLE) Channels in lobster ORNs are "gated" or opened, as in other neurons, in response to one of two applied stimuli: (1) voltage or (2) ligands such as odors, second messenger molecules, and neurotransmitters. Gap junction channels, regions of close apposition between the cell membranes of adjacent cells, which allow passage of larger molecules than just ionic species, are also hypothesized in olfactory neurons but undefined as yet in lobster ORNs. Voltage-gated channels are comprised of 4 homogeneous domains, ligand-gated channels of 5 heterogeneous domains, and gap-junction channels of 6 homogeneous domains. Curiously IP_3-gated channels (Chapter 4) fall into the family of ligand-gated channels by definition of their gating nature, although structurally these channels are more closely related to voltage-gated channels. (BOTTOM) A two-dimensional view of the channel protein demonstrates its main features: A **pore** region through which the ions conduct current from the external medium to the internal cytoplasm or vice-versa, a **selectivity filter** which contains a region that preferences a given ion over that of others based on molecular size and fast on-off binding reactions, a **gate**, reputedly hypothetical, which can be opened or closed in response, for example, to conformational changes induced by electrical charge migration, ligand binding, pH or temperature, and a **voltage sensor** which is susceptible to movement of electrical charges as a result of a change in membrane potential.

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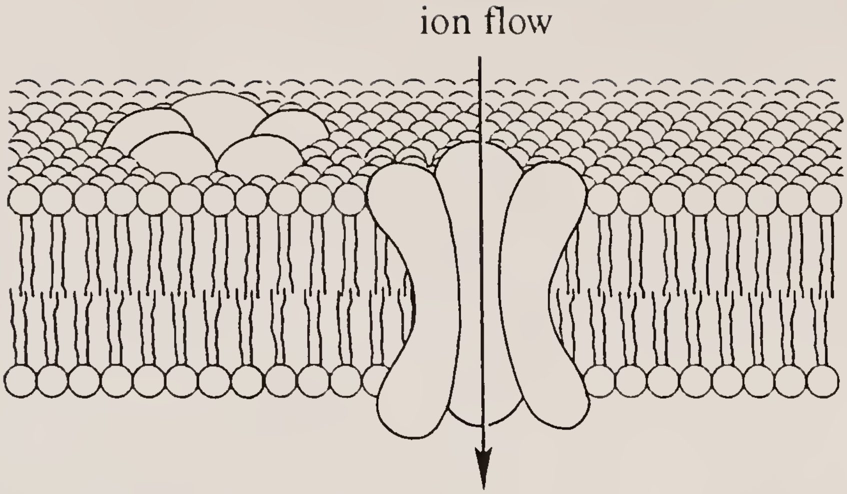
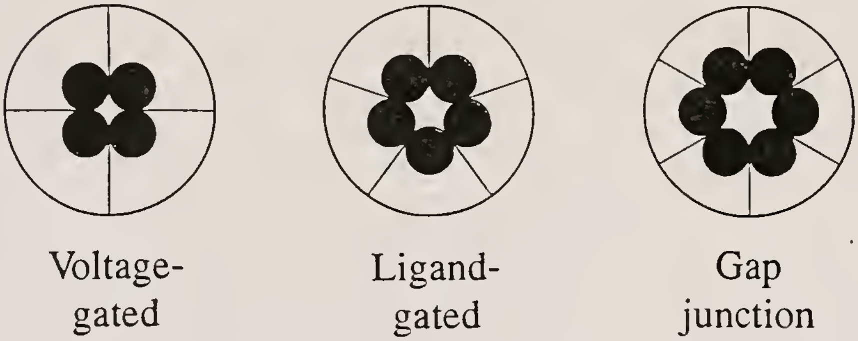
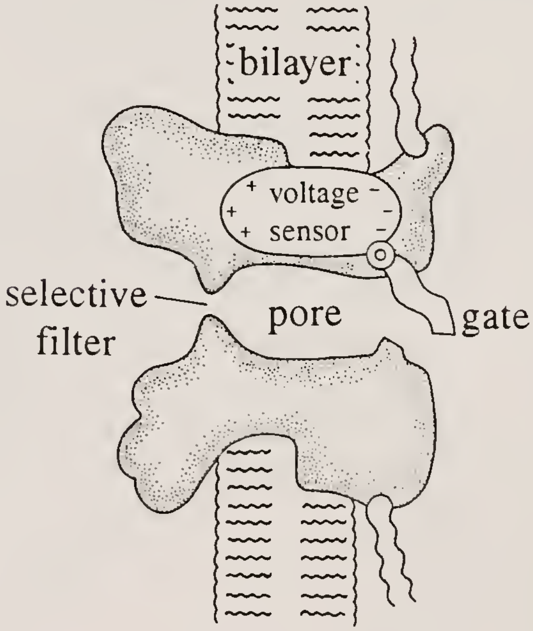

ion flow

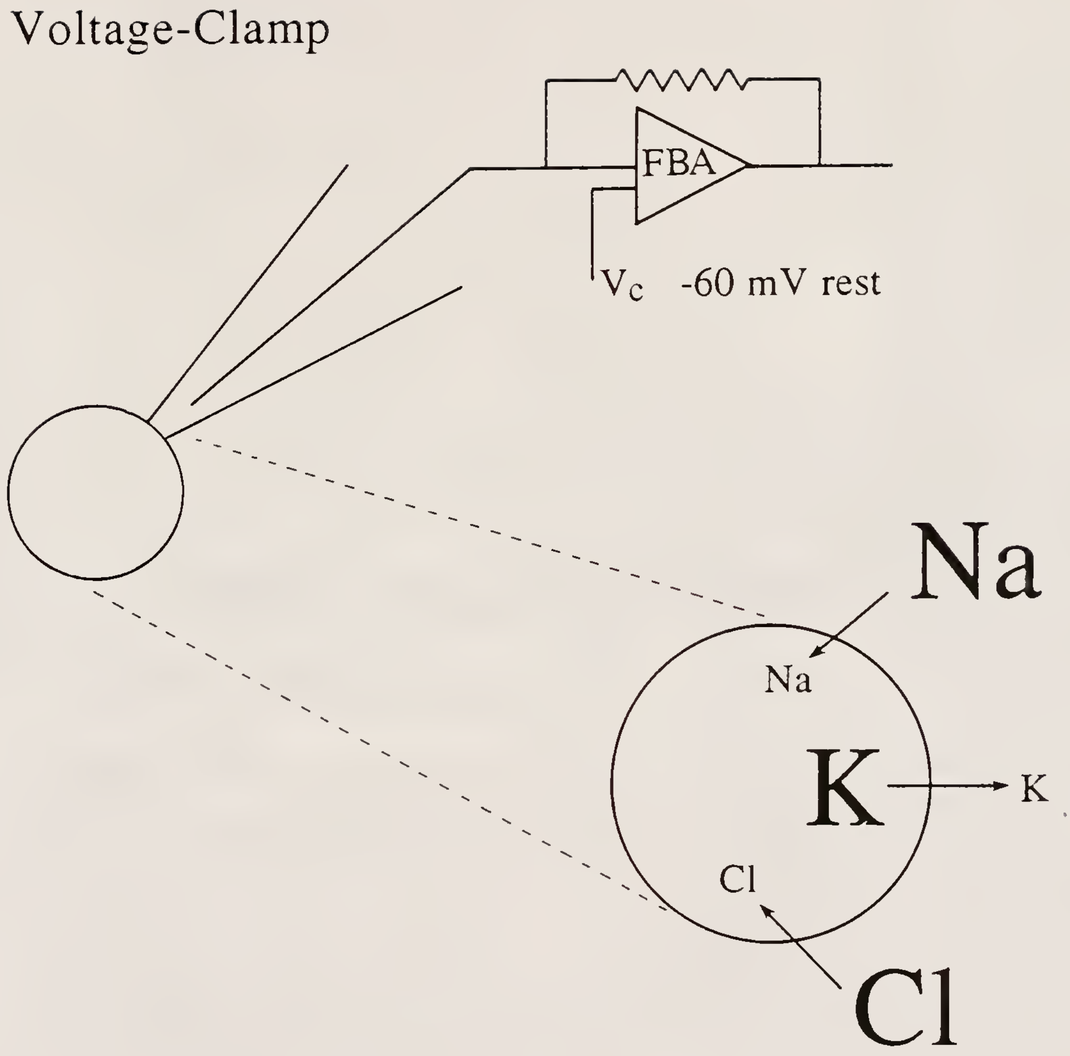

Voltage-gated
Ligand-gated
Gap junction


bilayer
+ voltage −
+ sensor −
+ −
selective filter
pore
gate

voltage-clamp recording, a feedback amplifier measures the difference between a set potential (command voltage) and the potential generated by the stimulated cell. This voltage differential is converted to a current and given back to the ORN to maintain the potential that was fixed on the amplifier (hence the term voltage-**CLAMP**; Figure 1-4). The amount of current given back to the ORN is used as a measure of what was received as the bioelectric signal from the cell. In some instances, a single electrode operates as the voltage sensing as well as current injecting device. In larger cells, one can use two separate electrodes, one for each function. Lobster ORNs are not big enough for the placement of two electrodes, so the one electrode has to flip-flop between the two functions.

The voltage-clamp method was first developed by Cole (1949) and Hodgkin et al. (1952) for use with the famous squid giant axon. The usefulness of the clamp stems from the fact that it is much easier to obtain information about currents measured from an area of membrane with a uniform, controlled voltage, than when the voltage is changing freely with time and between different regions of the membrane. The voltage-clamp was modified by Neher and Sakmann (1976), recent Noble laureates in medicine, for investigating the basic properties of ion channels. In order to directly measure the elementary events (ion flux through single channels) they achieved a reduction in background noise by

Figure 1-4. On either side of a biological excitable membrane there are ionic species of varying concentration, the primary ionic species being Na^+, K^+, Ca^{2+}, and Cl^-. The electrical and chemical gradient established by the relatively impermeable barrier determines the potential difference, close to -60 mV in lobster ORNs. The voltage-clamp measures the flow of charge through the protein pore of a channel; the bioelectric signal - current. The feedback amplifier (FBA) measures the difference between the set potential difference (command voltage; V_c) and the amount given by the stimulated cell as voltage. This given amount of voltage is converted to a current increment which is given back to the ORN to maintain the potential that was fixed on the amplifier. In most experiments, I commonly used a V_c = -60 mV to approximate the resting membrane potential in lobster ORNs.


Voltage-Clamp
FBA
V_c -60 mV rest
Na
Na
K
K
Cl
Cl

restricting the size of the recorded membrane to a small 10 μm^2 patch. They then electrically isolated the membrane patch from the rest of the cell by sealing the glass micropipette tightly onto the membrane. This is how the name **patch**-clamp recording was derived (Figure 1-5). It was only by accident that they discovered slightly negative pressure or suction created a molecular contact between the pipette and plasma membrane, which improved seal resistance into the gigaohm range (Neher et al., 1978).

Horn and Patlak (1980), Hamill and Sakmann (1981), and Hamill et al. (1981), discovered that patches could be removed from cells by retracting the glass pipette to achieve excised patches. Here patches are removed from their natural environment, either with the internal (inside-out configuration) or external side (outside-out configuration) of the membrane facing the outside bath, allowing optimal control of solution changes from either face of the membrane. Although retaining the cell in a cell-attached recording configuration permits measurements of a process with the least disturbance of the intact cell, sometimes more experimental control is required. Alternatively, strong suction can be applied while the pipette is still attached to the cell membrane to rupture the patch and create a whole-cell configuration, whereby the ionic milieu of the cell interior and the membrane potential can be controlled, and the cell is left otherwise intact (Figure 1-5).

I have used each of these pipette configurations in my studies towards a different gain: (1) While in the cell-attached configuration, a bath applied agonist that evokes channel activity generally infers a cellular mechanism requiring a second messenger molecule. (2) Membrane impermeant probes can be readily applied to the cytoplasmic face of the membrane in the inside-out configuration. (3) While in the outside-out configuration, a ligand applied to the bath that evokes channel activity strongly suggests a directly gated channel mechanism. I also took advantage of a modification of the inside-out configuration (Chapter 5) called "patch cramming" as described by Kramer (1990), where one takes an inside-out configured patch containing a channel of interest and inserts the patch pipette into a second, recipient cell. The channel activity of the patch while inside the recipient cell can then be used as a probe to detect changes in intracellular second messenger production in a living cell. In order to acquire baseline odor responsivity of a neuron, and to monitor changes from unstimulated states, I chose to either voltage-clamp single ORNs sequentially or to rely on the diffusional properties of solutions over time by backfilling electrodes with test solutions and tip-filling them with control solutions (Chapters 4-5).

Figure 1-5. (TOP) Diagram of a neuron in an on-cell or **cell-attached configuration**. This configuration permits measurements of a natural process with the least disturbance of the intact cell. Strong suction can be applied while the pipette is still attached to the cell membrane to create a **whole-cell configuration**, whereby the ionic milieu of the cell interior and the membrane potential can now be controlled by the investigator, and the cell is left somewhat intact. The measured current is defined as a macroscopic current, comprised of the total summation of all ionic flux from the channels contained in the whole cell. In lobster ORNs, an odor-evoked outward current is due to an efflux of cations; an inhibitory odor response. An odor-evoked inward current is due to influx of cations; an excitatory odor response. (BOTTOM) Membranes can be removed from neurons by retracting the glass pipette to achieve excised patches. Patches can be removed from their natural environment, either with the internal (**inside-out configuration**) or external side (**outside-out configuration**) of the membrane facing the outside bath, allowing optimal control of the solutions bathing either face of the channel. I commonly used the inside-out configuration (as shown) in order to apply the membrane impermeant second messenger, inositol 1,4,5-trisphosphate (IP_3) to the inside face of a channel. By convention, an ion passing into the cell (out of the patch pipette) is defined as an **inward** open channel event (O) and is displayed as a downward deflection in unitary current level away from the closed state (C). An ion passing in the opposite direction is displayed as an upward deflection in unitary current level and is described as an **outward** open channel event

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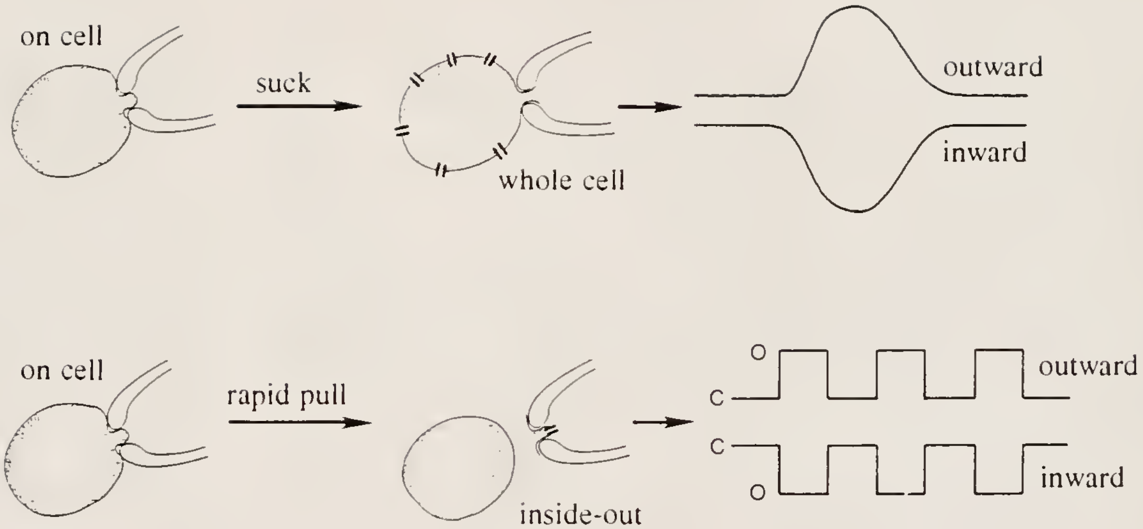
Patch Clamp


on cell
suck
whole cell
outward
inward
on cell
rapid pull
inside-out
O
C
outward
C
O
inward

The analysis of single channel events describes the behavioral details of the passage of a single ion species across the phospholipid bilayer of a neuron. An excellent source for the recording and analysis of currents from single ion channels is Wonderlin et al. (1990). Two excellent reviews by the noble laureates that first recorded unitary currents, provides information on the theory of channel behavior, as discussed in the next three pages of my dissertation, and can be found Rae and Cooper (1990), Neher (1992b), and Sakmann (1992). When quantifying the behavior of a single molecule, the channel protein, one generally calculates (1) the magnitude, (2) duration, and (3) order of channel events; all of which are random variables and none of which can be inferred by observing raw data. The information contained in channel events must come from a measurement of their distributions; statistics. The current thinking is that randomness of thermal motions underlies the dwell time of a channel. It is postulated that the bonds of the channel protein are vibrating, bending, and stretching on a picosecond time scale to achieve an open or closed conformation once an energy barrier is surmounted. The probability of surmounting such energy barriers to an open state (Pr_{open}) has been found to be dependent upon one or more natural stimuli for the channel in question: membrane potential, applied voltage; temperature; sensory inputs; second messenger, hormone, neurotransmitter, or intracellular ion concentration; or state of modulation, phosphorylation.

The first random variable, event magnitude, is typically calculated based on one of two distributions of unitary current amplitude: a point-by-point amplitude histogram or an event amplitude histogram. In the former, all sampled channel events are binned into assigned current levels, regardless of the state (open or closed) of the channel. In the latter, a histogram is constructed containing only amplitudes of events greater than 2.5 times the rise time (2.5 t_{rise}) duration of the event. The event amplitude is then measured by averaging the current level within a window beginning $\approx$ 1 t_{rise} after the opening and ending 1 t_{rise} before the end of the event. This window corresponds to the "flat" region of the event and excludes regions that may be distorted due to the finite t_{rise} of the recording instruments. In both types of distributions the mean and variance of the current magnitude can be determined by fitting the histogram with a Gaussian curve. An X-Y plot of the mean current as a function of membrane potential (voltage) can be used to generate the slope conductance of the channel, a measure of the degree of ion permeation. The greater the conductance of a channel, the faster the ions are flowing through the pore of the channel. Generally, low conductance channels have a higher degree of ionic interaction with charged amino acids inside the channel pore or between each other.

I chose to use the point-by-point amplitude histogram in my analysis because the number of channels in a patch can be detected with this distribution, Pr_{open} can be easily calculated, and presence of voltage-dependence can be determined. In a point-by-point amplitude histogram, the number of peaks minus one is generally the number of channels. If the peaks correspond to an integer function, then one is likely to be recording multiple openings of identical channels. If the peaks correspond to variable amplitudes, then one is likely to be recording heterogeneous multiple channels. The probability of opening, Pr_{open}, is defined as the total time a channel spends in the open state divided by the length of the recording. The integration of the area under the peaks in the amplitude histogram is used as an index of time. The presence of voltage-dependent channel opening can be determined by plotting Pr_{open} as a function of membrane voltage. Deviation from a zero slope would indicate voltage-dependent channel gating.

The second random variable is duration, the exponential distribution of random dwell times. One measures the average time (dwell) the channel spends in the open and closed state. The mean open time (t_o) or mean closed time (t_c) is dependent upon the rate constant (in sec^{-1}) leading out of that state. The mean dwell times are thus defined as $t_c = 1/\alpha$ and $t_o = 1/ß$ where C (closed) $\underset{\beta}{\overset{\alpha}{\rightleftarrows}}$ O (open). The movement between the O and C states requires a differential to describe its behavior, called the probability distribution function, which defines the intervals between the random channel events. The derivative, f(t), of this function is called a pdf or a probability density function, which is the function used to fit measured open or closed dwell times of a channel. Dwell times are reported in the form of a histogram, where each open or closed event is binned into a dwell time of a certain duration, and the exponential fit (f(t)) of the histogram provides the mean dwell time, usually reported in msec. The number of exponential components required to fit the distribution = the number of open or closed states of the channel.

Most membrane patches contain multiple channels and multiple states, which albeit complicates the analysis of channel behavior, provides a lot of information about the third random variable, order of channel events. Channel bursting, latency to first opening, change in mode or kinetic state, hibernation, subconductance, cooperativity, and steady-state flickering are all examples of complex channel behavior that can provide rich knowledge about the biology of a channel.

Specific Aims

I addressed five major questions during the course of my doctoral study. The resolution of each question helped shape the subsequent avenue of investigation as the following suggests, namely:

1. Can lobster (*Panulirus argus*) olfactory receptor neurons be sustained in primary cell culture?
2. Are the voltage-activated and odor-activated properties of the neurons altered by cell culture?
3. Do GTP-binding proteins (G-proteins) link binding of an odorant receptor to the generation of an odor-evoked current?
4. Are odor-evoked currents elicited by direct activation of an odorant receptor by an appropriate odor molecule or are currents evoked through a second messenger cascade?
5. What is the primary charge carrier of the plasma membrane IP_3-gated channels mediating excitatory transduction (inward currents) and can they be activated by other metabolites in the inositol phospholipid pathway?

CHAPTER 2
SUSTAINED PRIMARY CELL CULTURE

Introduction

Dissociated neurons in primary culture are providing useful models for a growing number of neurobiological studies (Benda et al., 1975; Sebben et al., 1990). Olfactory receptor neurons in many animals are long, thin bipolar neurons that terminate distally in a highly branched arbor of cilia or in an outer dendritic branch (Steinbrecht, 1969) that is suspected to be the site of chemosensory transduction (Lowe and Gold, 1990). Studying the physiology of transduction by directly patching the thin cilia or outer dendritic branches has been possible (Nakamura and Gold, 1987, Hatt and Zufall, 1990), but is technically difficult. In most instances, it is necessary to work with intact cells. In amphibians, some of the olfactory receptor cells contract when dissociated from the olfactory epithelium (Firestein and Werblin, 1989), thereby facilitating physiological analysis of transduction by allowing effective

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NOTE: This chapter has been accepted for publication and is reprinted with permission from Fadool, D.A., W.C. Michel, and B.W. Ache. 1991. Sustained primary culture of lobster (*Panulirus argus*) olfactory receptor neurons. Tissue & Cell 23(5): 719-732.

space clamping to characterize odor-activated currents and by allowing drugs introduced into the soma through the patch electrode to diffuse to the cilia. In other animals, however, dissociated receptor neurons retain their diffuse morphology and are less amenable to physiological analysis of transduction. In these instances, physiological analysis of transduction would be facilitated if, by placing the cells in culture, it were possible to obtain morphologically more compact cells that still retained their responsiveness to odors.

Lobster olfactory neurons have one of the longest dendrite to soma distances reported for olfactory receptor cells in any organism (*ca.* 1 mm; Grünert and Ache, 1988). In order to study the physiology of transduction in these cells, it would be particularly useful if the cells could be sustained in primary culture in a morphologically more compact form. Most tissue culture protocols, however, have been designed for mammalian systems (especially human, rat, mouse, and rabbit). Although techniques for culturing insect tissue are now commercially available, culturing techniques for other invertebrate nervous tissues are less well established. In particular, crustacean tissue culture is just in its initial stages of development (Fainzilber et al., 1989). Only recently have techniques for culturing stomatogastric ganglion (Graf and Cooke, 1990; Krenz and Fischer, 1990), peptidergic neurons (Cooke et al., 1989), adipose tissue (Van Beek et al., 1987), proprioceptor organs (Hartman et al., 1989), and ovarian tissues (Fainzilber et al., 1989) been reported using crustacean species. The above protocols reported widely varying conditions suggesting that a systematic test of culture parameters may be necessary for each crustacean species.

In order to better study the electrophysiological properties of lobster olfactory receptor neurons, therefore, I developed techniques to sustain the cells in primary culture. In this chapter nine culture parameters are described that were systematically tested to establish an *in vitro* model which most closely approximated the osmolarity and salt composition of the lobster's physiological fluid; haemolymph; approximated known physical parameters, such as temperature; and which included supplements known to have effects on nerve outgrowth. In this chapter are reported the conditions that allow cells harvested from lobster olfactory organs (antennules) to survive in primary culture for 23 days. Cultured cells are more compact than cells *in vivo*, and most importantly, are electrically excitable and odor sensitive; that is, they bear the physiological markers of olfactory neurons. The potential these cultured cells have for studying the physiological process of olfactory transduction is discussed.

Methods

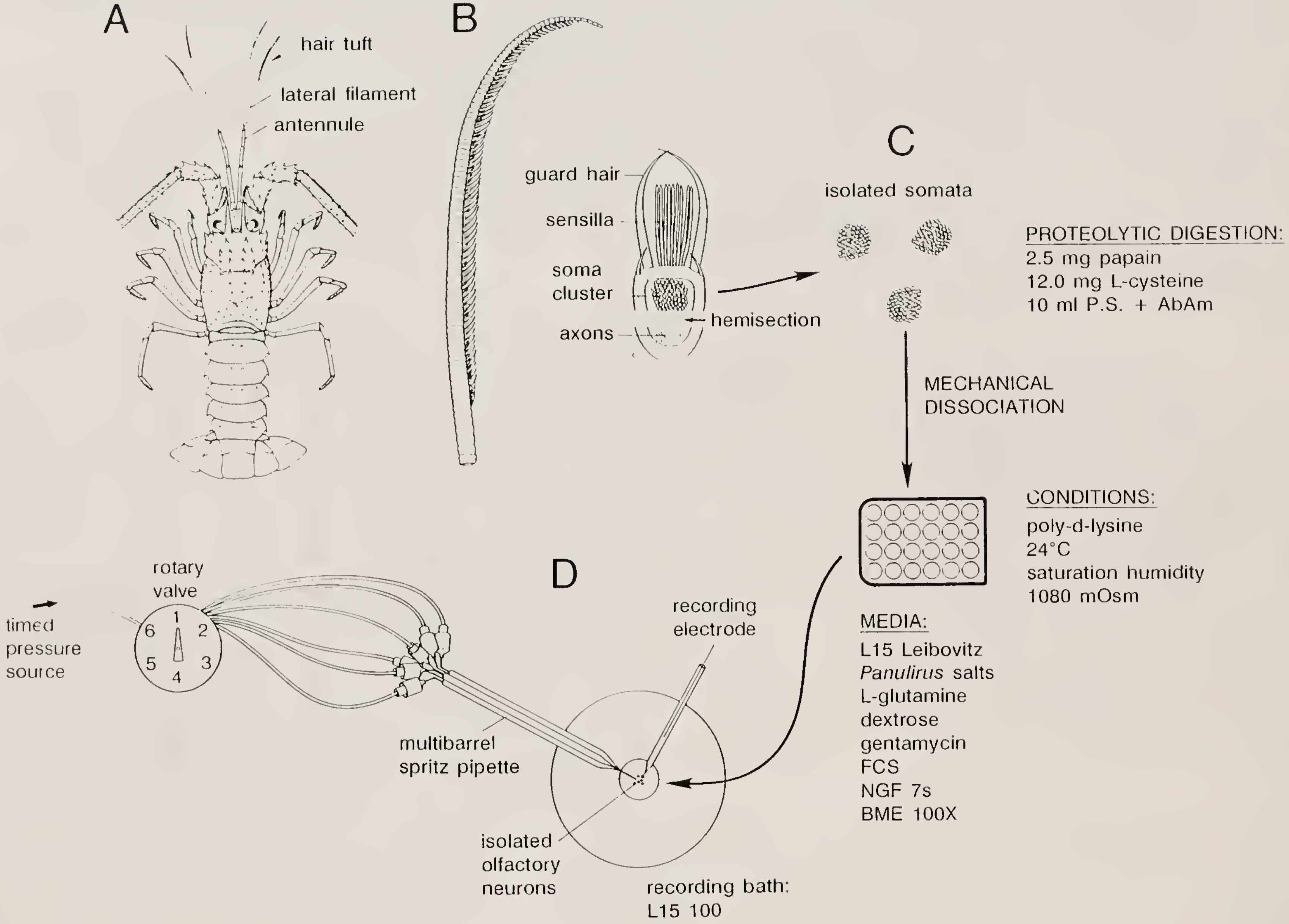
Animals

Specimens of the Caribbean spiny lobster, *Panulirus argus*, were collected from the Florida Keys and maintained in an open circulating sea water system. Animals were fed a mixed diet of frozen fish, squid, and shrimp.

Tissue Preparation

The olfactory organ (distal third of the lateral antennular filament) was excised from intermolt animals (Figure 2-1, A-C) and washed in 10% Listerine in *Panulirus* saline (PS, see solutions) containing 1% penicillin, streptomycin sulfate, and amphotericin B as Fungizone (Gibco; AbAm). The organ was cut into sections three annuli long, hemisected, and transferred to fresh PS + AbAm. Soma clusters of the olfactory (aesthetasc) receptor cells, which literally fill the lumen of the organ, were removed from the hemisection with a sterile 26 gauge syringe needle, washed repetitively with PS + AbAm, and transferred to fresh PS + AbAm. Repetitive washes were critical to eliminate contamination, presumably from epiphytes on the exoskeleton. The isolated soma clusters were then incubated for 50 min at 80 rpm on an orbital shaker in 0.2 micron filter-sterilized: 10 ml PS + AbAm containing 2.5 mg papain and 12 mg L-cysteine.

Figure 2-1. Procedures for establishing lobster olfactory receptor neuron (ORN) cultures. A) The olfactory organ is comprised of chemosensory sensilla that are located on the distal third of the lateral antennular filament. B) The organ is excised from the spiny lobster, and cut into 3 annuli section, one of which is shown in cross section. Note that the clusters of olfactory receptor cell somata literally fill the lumen of the antennule once the section is hemisected and axons removed. C) Clusters of olfactory receptor cell somata are then isolated, proteolytically treated, mechanically dissociated, and plated on substrate coated coverslips for cell culture. D) ORNs on coverslips are removed from culture for electrophysiological recording. The odors are delivered to the cells by "spritzing" from a 6-chambered multibarrel pipette, connected by way of tycon tubing to a rotary valve in series with a picospritzer to deliver a 120 msec pulse of odorant onto the ORN. AbAm = antibiotic-antimycotic 1% solution, PS = *Panulirus* saline, FCS = fetal calf serum, NGF 7s = nerve growth factor 7s, BME 100X = basal minimum essential vitamins.


A
hair tuft
lateral filament
antennule
B
guard hair
sensilla
soma cluster
axons
hemisection
C
isolated somata
PROTEOLYTIC DIGESTION:
2.5 mg papain
12.0 mg L-cysteine
10 ml P.S. + AbAm
MECHANICAL DISSOCIATION
CONDITIONS:
poly-d-lysine
24°C
saturation humidity
1080 mOsm
MEDIA:
L15 Leibovitz
Panulirus salts
L-glutamine
dextrose
gentamycin
FCS
NGF 7s
BME 100X
D
timed pressure source
rotary valve
1
2
3
4
5
6
multibarrel spritz pipette
recording electrode
isolated olfactory neurons
recording bath:
L15 100

Cell Cultures

Proteolytic digestion was stopped by replacing the enzyme solution with modified low glucose L-15 media (50 ml L-15 Stock, 50 ml of 1.6X normal concentration of PS, 0.6 g dextrose, 0.029 g L-glutamine, and 0.01% gentamicin). Cells were dissociated by trituration using a sterile 23 gauge needle and plated on poly-D-lysine hydrobromide (MW 49,300-53,000) coated glass 12 mm coverslips (2.5-5.0 $\mu g/cm^2$) at a cell density of 12 x 10^4 cells/ml (per 2 cm^2 well). Cells were placed in the dark and allowed to adhere to the coverslips for 2 hr without agitation. After this period, 6% fetal calf serum (FCS) or 10% lobster haemolymph was added to each well. In experiments requiring haemolymph supplementation, the blood extraction procedures of Fadool et al., 1988 were followed. Haemolymph osmolarity was measured in 200 μl samples by freezing point depression (Osmette #2007, Precision Systems, MA). Only one-half of the media was changed every fourth day to allow accumulation of any required neurotrophic factors. Cells were maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) inside a low-range temperature incubator (Hotpack) at 24°C.

Experimental Culture Conditions

Nine culture parameters were systematically varied over 22 cultures and their effect on yield and longevity, if any, noted: (1) osmolarity (917 to 1079 mOsm), (2) temperature

(20 to 28°C), (3) humidity (60% to complete saturation), (4) HEPES buffering, (5) serum supplementation at 2, 12, or 24 hr after plating, (6) basic minimal essential (BME) vitamin, L-glutamine, and nerve growth factor 7s (NGF-7s) supplementation, (7) substrate (glass, plastic, poly-D-lysine, laminin, collagen, or haemolymph clots), (8) length of proteolytic digestion (20 to 60 min), and (9) duration of animal holding (1 to 8 wk). Cell counts were made daily of a permanently marked field of view in each well of a 24 well plate.

The effect of supplementing olfactory neuron culture media with media preconditioned with lobster brain tissue was measured in a separate series of experiments. Either entire brain or the olfactory and accessory neuropils were isolated from the anterior region of cold anesthetized lobsters. Tissues were rinsed in 3 volumes of PS + 5% AbAm and then diced into fine pieces in a small volume of modified L15 media. Whole tissue slices were portioned into 1 ml modified L15 media, supplemented with 40 μl of FCS, and continually agitated at 50 RPM on an orbital shaker. Conditioned media was collected after 24 and 60 hr and used to supplement olfactory neuron cultures at 50% (50 L15 media: 50 conditioned media) and 30% (70 L15 media: 30 conditioned media) concentrations. *In vitro* brain tissue fragments were maintained for 2 wk by changing one-half media every third day and re-supplementing with 35 μl FCS.

Electrophysiology

The cells were patch-clamped in the whole-cell configuration, using an integrating patch-clamp amplifier (Dagan 3900). The signal was filtered with a low pass Bessel filter at 5 kHz and digitally sampled during odor stimulation every 4 msec. Acquisition and subsequent storage and analysis of the data was done using pCLAMP software (Axon Instruments). Neurons were viewed at 40X magnification under Hoffman optics. Patch electrodes of 1.8 mm O.D. boralex glass were fire polished to a tip diameter of approximately 1.0 μm (Bubble number 4.8; Mittman et al., 1978). High resistance seals (R_e = 8.0 to 14 GΩ) were formed by applying gentle suction to the lumen of the pipette. Odorants (pipette concentration 10^{-3}M) were delivered to the cells from a multibarrel glass micropipette (Frederick haer & Co.) coupled to a pressurized valve system (120 msec pulses; Picospritzer; General Valve Co.) via a 6-way rotary valve (Figure 2-1D).

Solutions

All salts used in preparing *Panulirus* saline and modified Liebowitz L15 Media were obtained from Sigma Chemical Co. NGF-7s from mouse submaxillary glands was obtained from Boehringer Mannheim. The patch electrode solution consisted of (in mM) 30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 180 K-acetate, and 696 glucose; pH adjusted to 7.0 with 5N KOH. PS consisted of (in mM): 458 NaCl, 13.4 KCL, 9.8 $MgCl_2$, 13.6 $CaCl_2$, 13.6 Na_2SO_4, 3 HEPES, and 2 glucose; pH adjusted to 7.4 with 1N NaOH. Modified L15 Media was prepared as follows: 50 ml Liebowitz L15 Stock, 50 ml of 1.6X normal concentration of PS, 0.6g dextrose, 0.026 g L-glutamine, 1% BME (basic minimal essential) vitamins (Sigma), and 0.01% gentamicin. Solutions of substances tested as odors were either (1) a 100-fold dilution of a complex mixture prepared from TetraMarin (TET), a commercially available flake fish food, made into an aqueous extract by homogenization of 2g dry flakes into 60 ml saline, followed by low speed centrifugation to remove particulates then filtration with Whatman #3, or (2) 10^{-3}M solutions of taurine, betaine, ascorbic acid, proline, glycine, cysteine, AMP, TMAO, or arginine, prepared daily in the modified L15 media. Odor concentrations are reported as the pipette concentration and not the absolute test concentration reaching the cell. The absolute concentration was estimated to be 91.5% of the pipette concentration, based on a method for determining stimulus concentration using the steady-state K^+ permeability of neurons (Firestein and Werblin, 1989).

Results

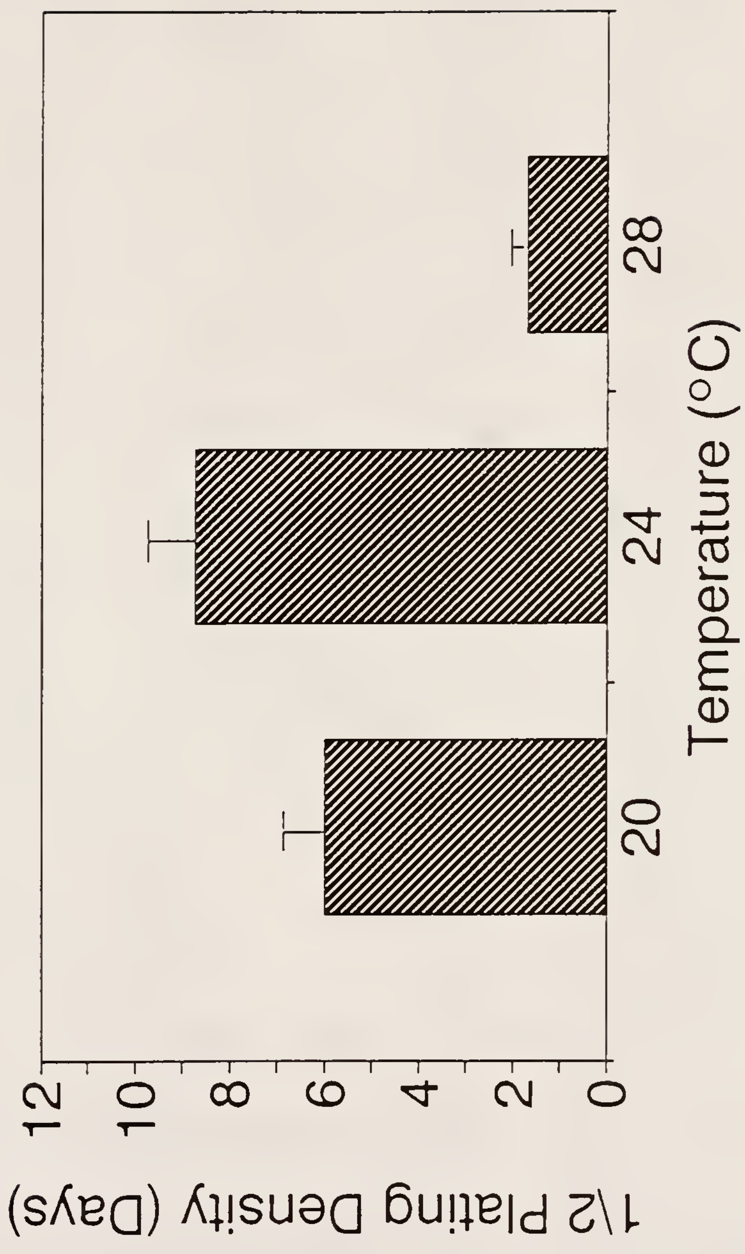
Optimal Culture Conditions

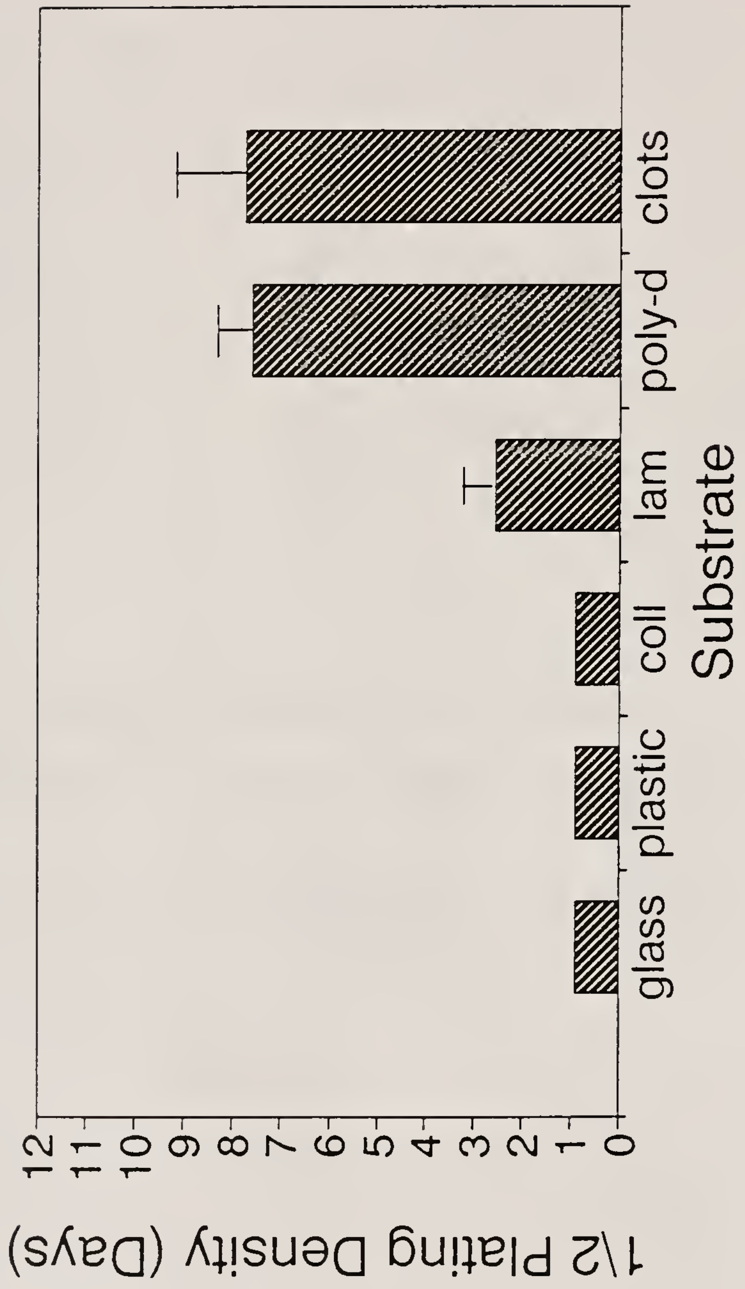
Optimal culture conditions were determined based upon measurement of cell survival; time in days until 1/2 the original plating density. Cells survived better at saturation humidity than at 60 and 80% humidity, where cell densities were reduced 1 wk after plating by 38 and 21 percent, respectively, in comparison with 100% saturated controls. Of the three temperatures tested (20, 24, and 28°C), the cells survived longest at 24°C (Figure 2-2A). Survival at 24°C was significantly longer than at the next best temperature (20°C).

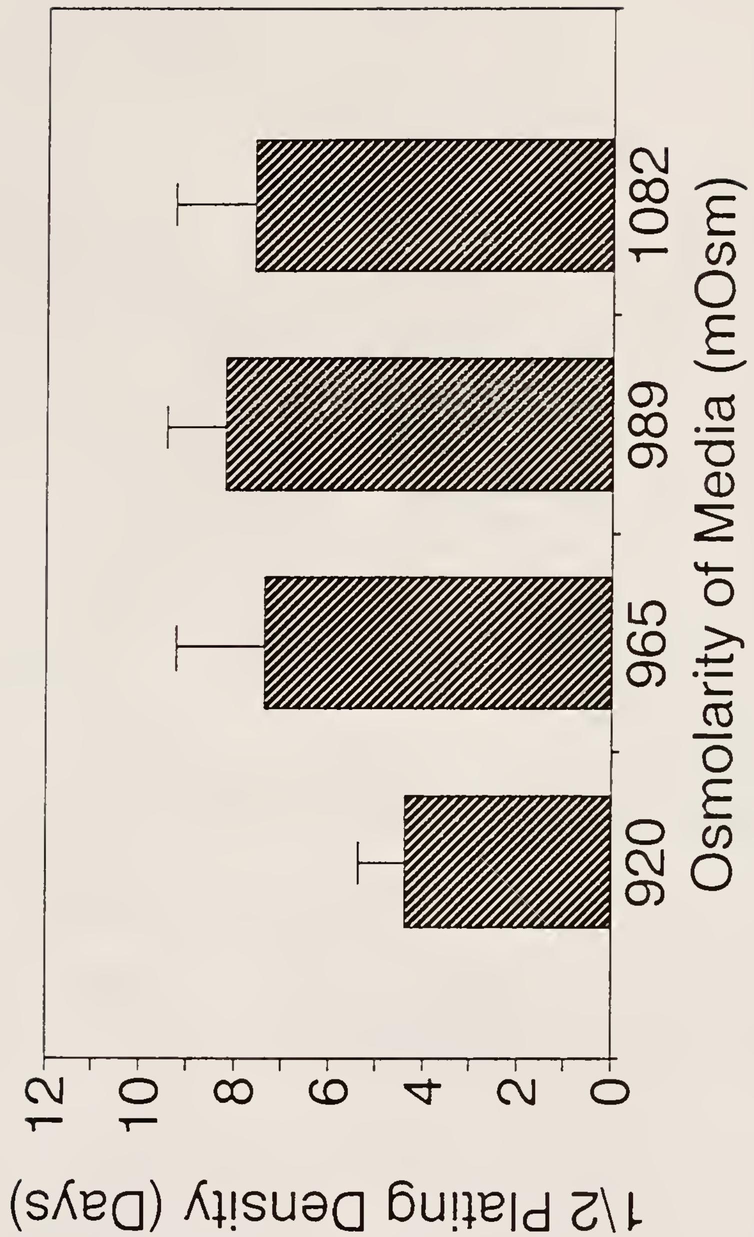
Cells did not adhere to untreated glass coverslips, collagen or laminin substrates, and adhered with little neurite outgrowth to commercial plastic tissue culture wells or dishes (Corning and Falcon #1008, respectively). Cells that did not adhere to an appropriate substrate, or as a population commence neurite extension, died within 36 hr. Cells sprouted processes with optimal survival when plated on poly-D-lysine coated glass coverslips or when grown on haemolymph clots (Figure 2-2B). Cells that adhered to poly-D-lysine substrate or clots did so immediately after plating in the absence of FCS. Process outgrowth and/or extension was observed as early as 10 min after plating. Cells did not adhere uniformly to the substrate when FCS was provided upon plating (vs. 2 to 12 hr later) nor when cells were kept under room light or continually agitated.

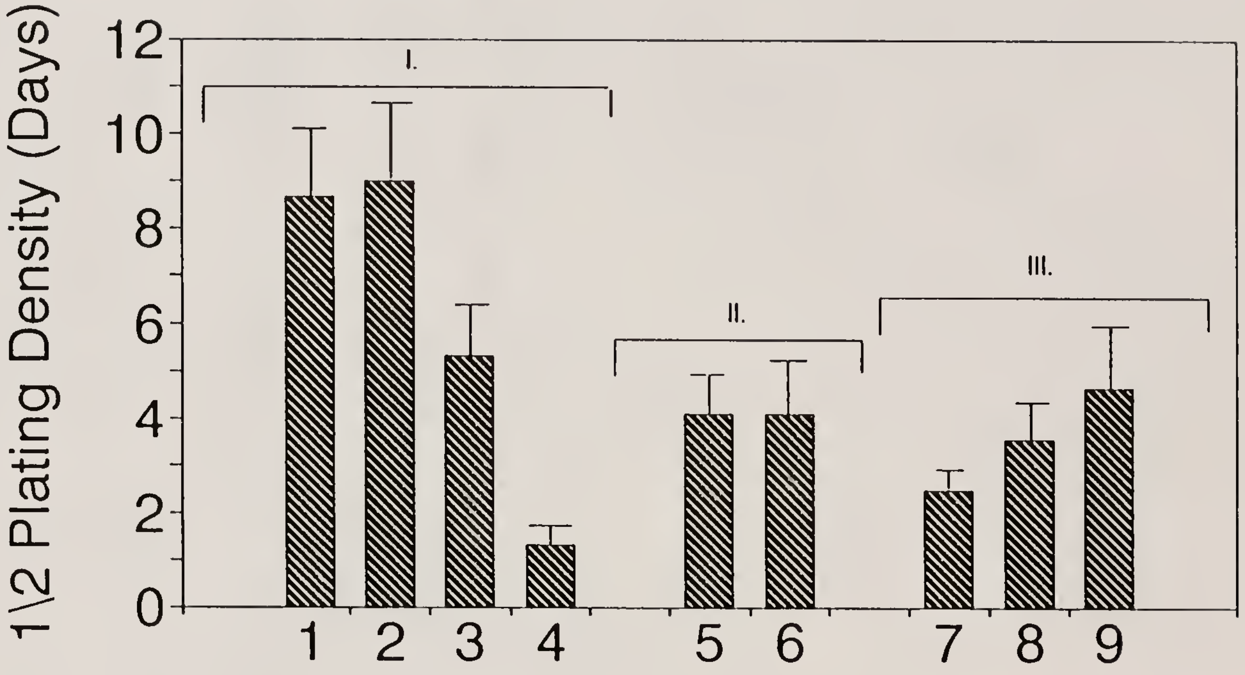
Cells survived equally well between 965 and 1082 mOsm, the highest of the four osmotic conditions tested. Survival was reduced significantly at 920 mOsm, the lowest of the four osmotic strengths tested (Figure 2-2C). Actual

Figure 2-2. Influence of A) temperature, B) substrate, C) osmolarity, and D) supplementation on ORN longevity. Plotted data are mean survival of neurons (days ± SEM) for 24 wells of at least 2 cultures per condition. Supplementation conditions referred to in D) are: Experiment I - (1) FCS at 2hr + Nerve Growth Factor (NGF 7s), (2) FCS at 2 hr - NGF 7s, (3) FCS at 12 hr - NGF 7s, (4) FCS at 24 hr - NGF 7s. Cells were maintained at 24°C in a humidified incubator; Experiment II - (5) + 3mM HEPES, (6) - 3mM HEPES. Both treatments were tested in the presence of FCS. Cells were maintained at 20°C at less than saturation humidity; Experiment III - (7) cells cultured from animals held greater than 8 wk, (8) held less than 3 wk, (9) held less than 3 wk + BME vitamin supplemented. Cells were maintained at 24°C at less than saturation humidity. ORN = olfactory receptor neuron, FCS = fetal calf serum.


1\2 Plating Density (Days)
12
10
8
6
4
2
0
20
24
28
Temperature (°C)


1\2 Plating Density (Days)
0
1
2
3
4
5
6
7
8
9
10
11
12
glass
plastic
coll
lam
poly-d
clots
Substrate


1\2 Plating Density (Days)
0
2
4
6
8
10
12
920
965
989
1082
Osmolarity of Media (mOsm)


1\2 Plating Density (Days)
0
2
4
6
8
10
12
I.
II.
III.
1
2
3
4
5
6
7
8
9

haemolymph osmolarity of intermolt animals was found to be 1079 ± 37.5 mOsm (SEM, N=4). Cells demonstrated a marked requirement for BME vitamins, L-glutamine, and FCS supplementation (Figure 2-2D). Cell survival was maximal when FCS was supplied 2 hr after plating (Figure 2-2D - 1,2). Cell density was reduced by one-half when cells were deprived of FCS for as short as 12 hr (Figure 2-2D - 3); few to no cells survived 24 hr of deprivation (Figure 2-2D - 4). The addition or omission of HEPES (Figure 2-2D - 5,6) or NGF-7s (Figure 2-2D - 1,2) had no gross effect on either longevity or neurite outgrowth. When antennular tissue was taken from animals held 8 wk or longer as opposed to animals held no more than 3 wk (Figure 2-2D - 7,8), culture plating density dropped by one-third within the first hours of plating, with few cells extending processes. BME vitamin supplementation increased cell survival when using animals held less than 3 wk (Figure 2-2D - 9).

Cells could be maintained in culture up to 23 days, using the derived optimal conditions. Media preconditioned with CNS tissue, added in amounts up to 30 or 50% of normal olfactory neuron culture media, had no significant effect on longevity of olfactory neuron cultures compared to control, non-conditioned cell cultures in the observed 23 days. This was true whether the media was conditioned for 24 or 60 hr, or whether it was derived from either whole brain, or the isolated olfactory and accessory neuropils.

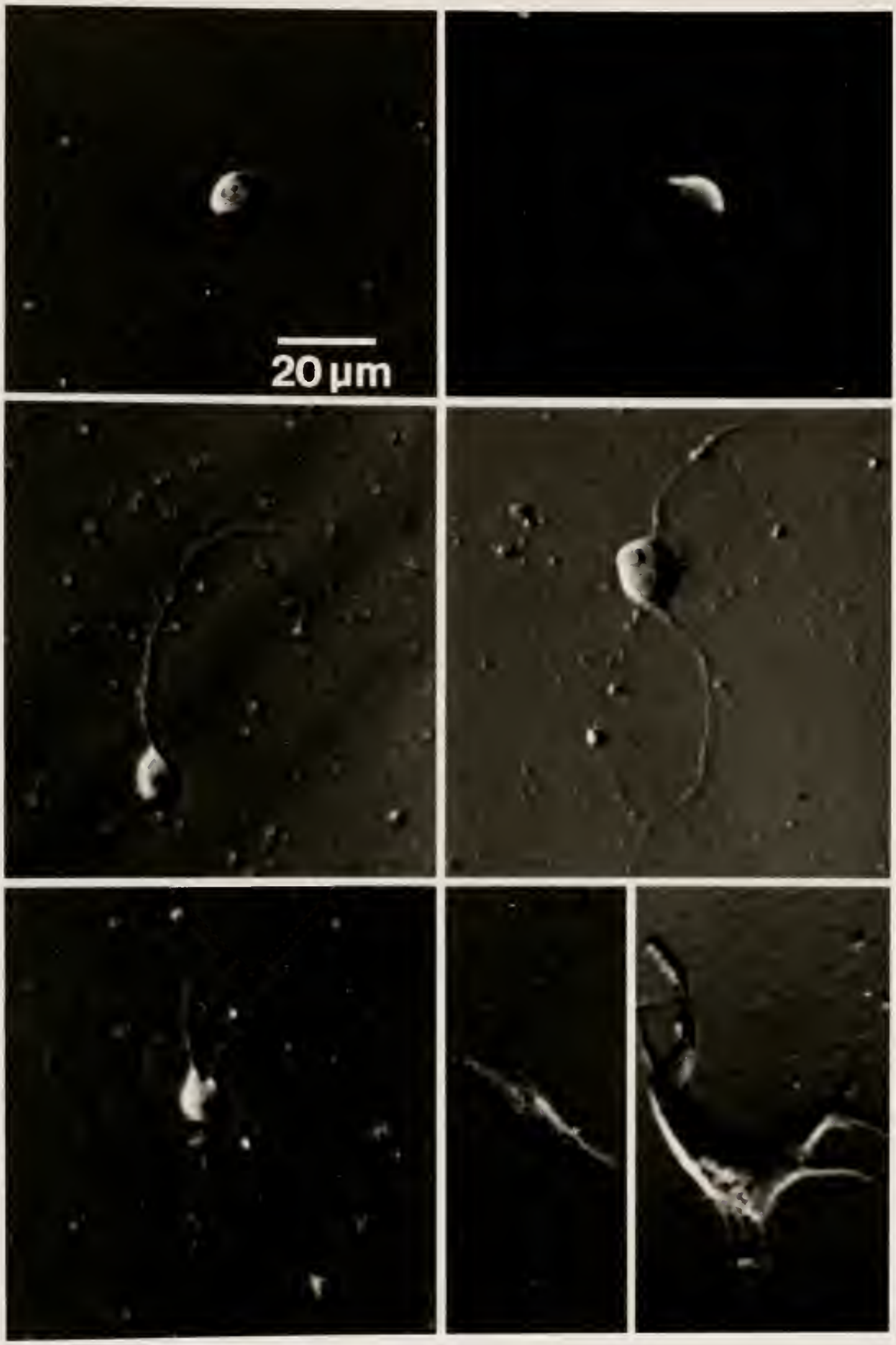
Morphology of Cell Types and Neurite Outgrowth

Five morphologically distinct types of "neuron-like" cells could be defined based on the number and type of processes: (1) soma only, (2) bud, (3) unipolar, (4) bipolar, and (5) multiprocess (Figure 2-3). Soma only cells were spherical in shape and underwent no apparent process formation. Bud cells possessed a single short process, less than 5 μm long, typically 3-4 μm wide, which did not branch. Unipolar cells bore a single long process, greater than 75 μm in length, often unbranched and thin. Bipolar cells always possessed two equal length processes; the majority of which were unbranched. Multiprocess cells produced three to five processes that were prominently arborous and of nonuniform length and width. In all instances, soma diameters ranged from 8 to 16 μm with processes, when present, between 3 and 160 μm long. In all cultures, larger, "non-neuron-like" cells with somata greater than 20 μm in diameter were also observed and comprised approximately 5% of a given cultured population. The larger cells were either fusiform or flat (Figure 2-3). These larger cells could be selectively removed by withholding FCS supplementation for 12-24 hr after plating.

Electrophysiology

All smaller cells (8-16 μm) tested were electrically excitable; voltage elicited a transient inward current followed by a sustained outward macroscopic current, which

Figure 2-3. The morphology of cells observed in lobster olfactory cultures. (A-E) "Neuron-like" cell types, 8-16 μm in diameter; A) soma only, B) bud, C) bipolar, D) multiprocess, and E) unipolar. (F-G) "Non neuron-like" cell types, greater than 20 μm in diameter; F) fusiform and G) flat. Magnification 780X.


20 µm

activated concurrently at *ca* -30 mV (Figure 2-4). In a population of 50 cells the average peak amplitude of the voltage-activated inward and outward macroscopic currents, when the cells were stepped from -60 (rest) to +30 mV, was -322.3 ± 27.9 pA (SEM) and 756.9 ± 30.0 pA (SEM) respectively. The ionic basis for these conductances was not determined. In contrast to the smaller diameter cells, the larger (≥ 20 μm) cells were not measurably electrically excitable, and had ohmic current\voltage relationships over the range of membrane potentials tested from -60 mV to +30 mV (n= 11). This dichotomy between the smaller and larger cells was mirrored in the sensitivity of the two types of cells to odors. None of the large cells tested responded to applied odors (n= 14), whether the odor was a single compound or the complex mixture (TET). In contrast, 28 of 50 (56%) smaller cells, each tested with one of a variety of single compounds (taurine, betaine, ascorbic acid, proline, glycine, cysteine, AMP, TMAO, or arginine) responded with either an inward or outward current (Figure 2-5). The collective cell population did not all respond to the same single compound. The latency of the odor-evoked current was phase locked to the application of the odor stimulus. The half-peak duration of the odor-evoked current was approximately 3 to 4 s. Although dose-response relationship was not formally tested the amplitude of the current increased with concentration over the range of 10^{-6}

Figure 2-4. Voltage-activated currents in a cultured ORN. A) Whole-cell macroscopic currents under voltage-clamp conditions. The cell was held at -60 mV and stepped to +30 mV in 10 mV episodes. B) Note that the IV plot of the cell displays the conductances which characteristically activated around -30 mV (+ = inward current, □ = outward current). All cells with "neuron-like" morphology showed similar IV relationships.

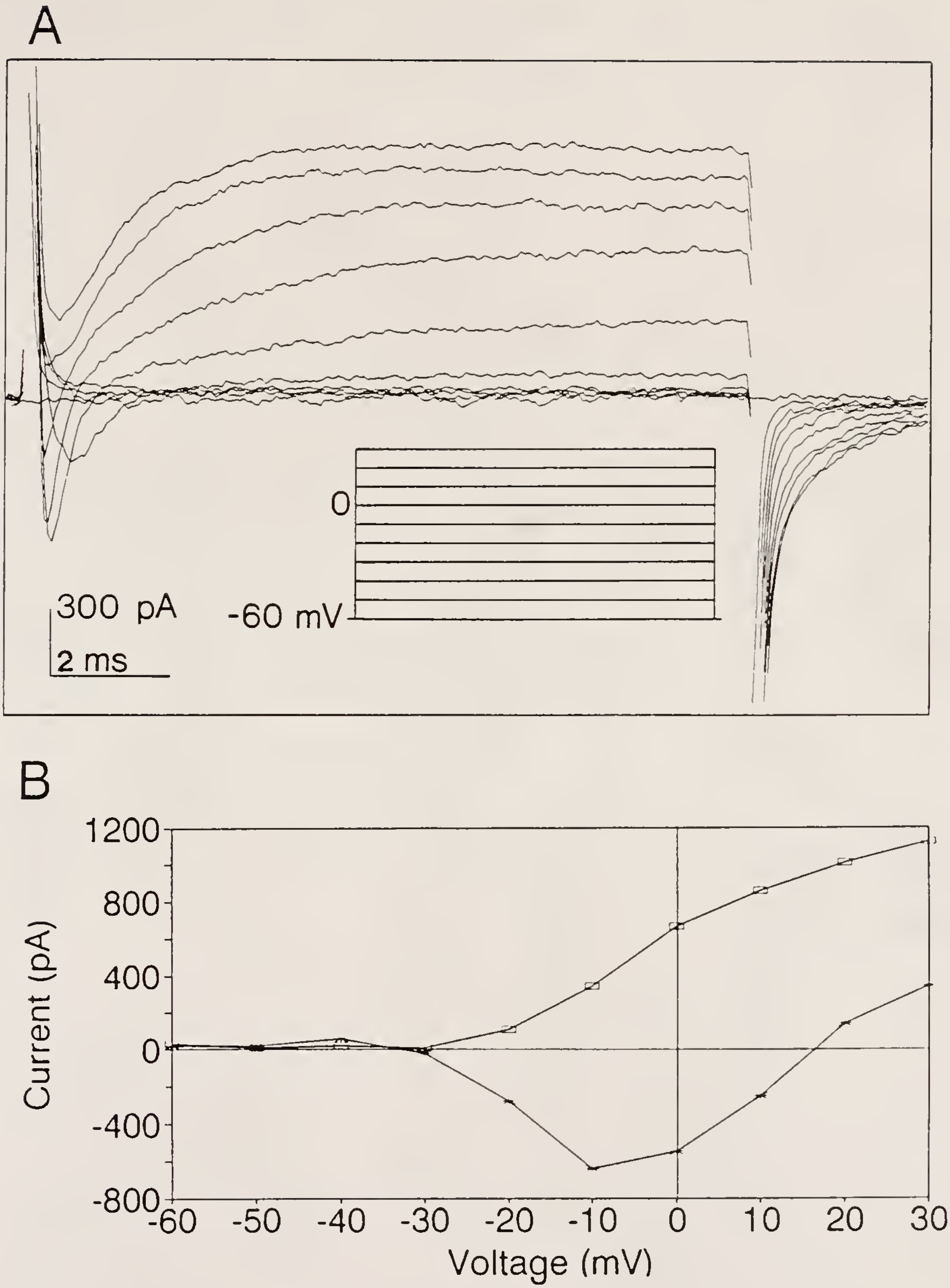
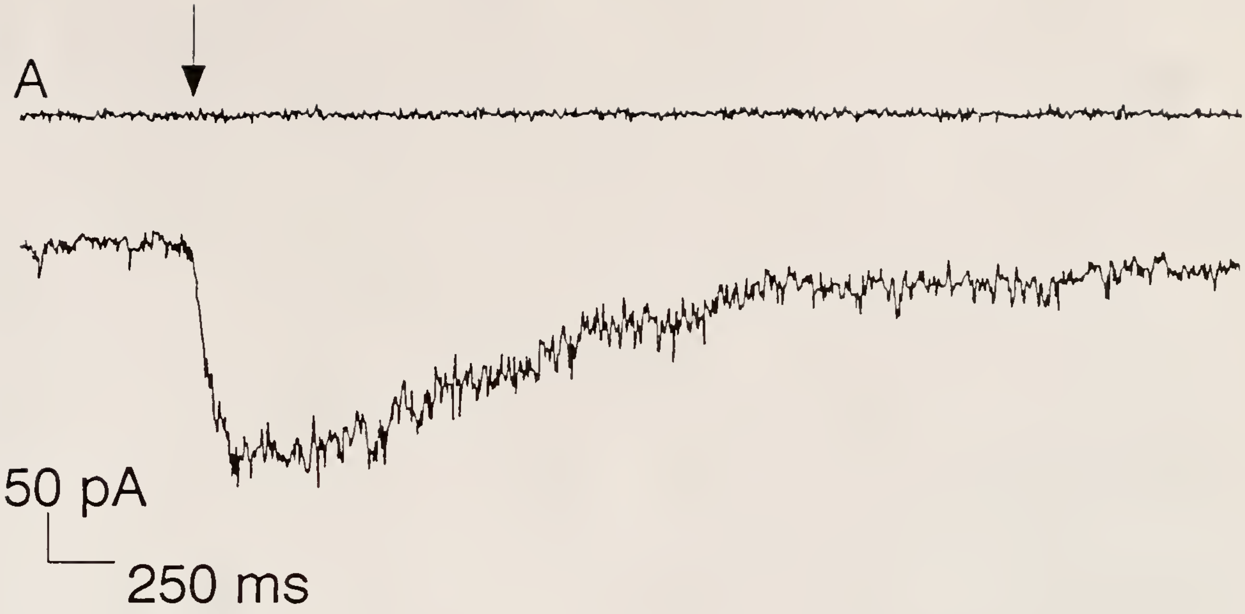
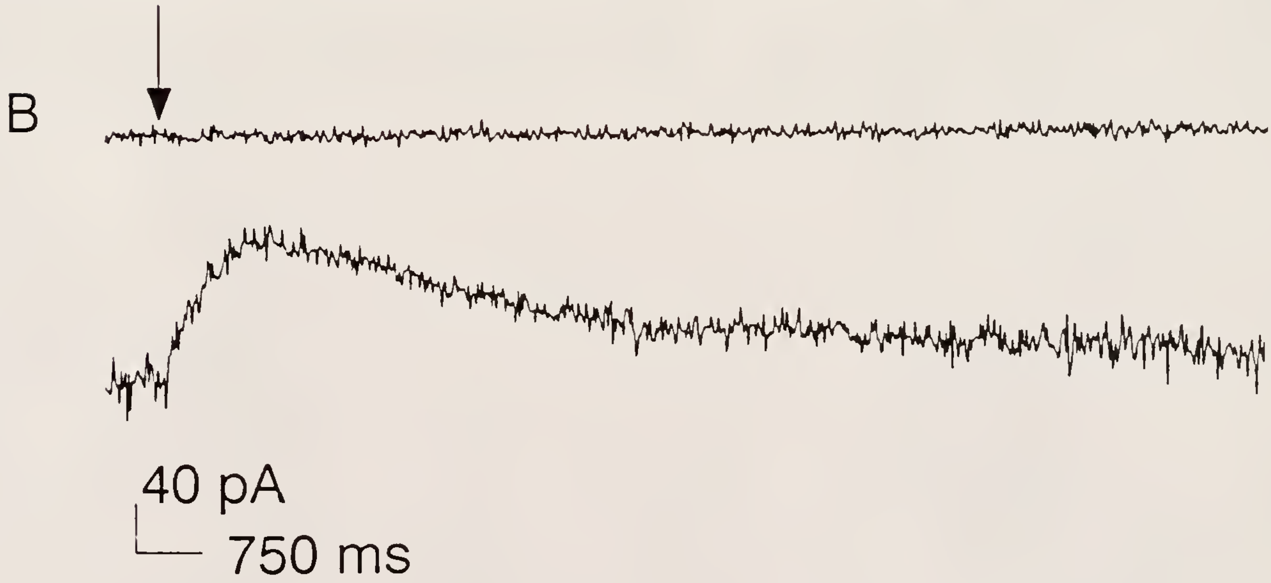

A
300 pA
2 ms
0
-60 mV
B
1200
800
400
0
-400
-800
Current (pA)
-60
-50
-40
-30
-20
-10
0
10
20
30
Voltage (mV)

Figure 2-5. Response of cultured ORNs to A) a 1000X dilution of TET extract and B) 10^{-3} M glycine. Control trace for each cell shows the response to a L15 media blank. Holding potential = -60 mV. Arrow = start of 120 msec odorant pulse.


A
50 pA
250 ms


B
40 pA
750 ms

to 10^{-2}M. An odor-evoked current could be distinguished from background noise at a 10^{-8}M odorant concentration in one cell tested for threshold. Eight out of ten cells tested (80%) with three to five odors, responded to at least one odorant. None of these cells responded to all five compounds; typically they responded to only one or two. When cells responded to more than one compound, the magnitude of the response varied across the effective tested compounds and also between different cells tested. In no instance did identical application of only culture media from one barrel of the stimulus delivery pipette elicit any measurable current. Similar control (flat) traces were recorded when the picospritzer pressure was turned off, and continuous cell baseline current was monitored.

Discussion

Under optimal conditions of temperature; humidity; osmolarity; appropriate substrate; serum, sugar, vitamin, and amino acid supplementation, olfactory neurons could be sustained for 23 days in primary culture, with continual neurite outgrowth during this period. The culture consisted of two fundamentally different types of cells that could be initially classified according the soma diameter. Based upon their selective responsiveness to odors and current, the smaller diameter cells (8 to 16 μm) would appear to be neurons. Since the lobster chemosensory organ is almost exclusively comprised of olfactory receptor cells (Grünert and Ache, 1988), the cultured system is developed from an enormously enriched starting population of olfactory neurons, with proportionately few neurons of other types (i.e. non-olfactory chemoreceptors and mechanoreceptors). Given an average of 350 receptor neurons per soma cluster and an average of 15 soma clusters per annulus (Grünert and Ache, 1988), the 90 annuli used per culture should yield 472,500 neurons. Based upon hemocytometer counts, I estimate that 288,000 neurons are harvested and plated per culture, which indicates an approximate total yield of 61 percent. Some loss would be expected from mechanical dissociation, pipette transfer, initial AbAm washes, and removal of soma clusters from connective tissue adjoining the cuticle during isolation. Based upon the yield alone, it is plausible to conclude that a large percentage of the small cells are olfactory neurons. Further evidence, however, is required to definitively prove that these cells originate from the olfactory (aesthetasc) sensilla.

Biologically relevant odors for aquatic animals are small molecules such as the amino acids used in this study (Carr et al., 1984). Since many of these molecules also have broad biological activity on cells (Carr et al., 1984), a systematic study of odor responsiveness of cultured cells as it compares to that of cells *in situ* is necessary in order not to falsely confuse broad action of these molecules with their actions as odorants. This initial test of odor sensitivity in cultured cells does demonstrate a degree of selectivity. Both types of odor-evoked currents are recorded in culture and cells display odor sensitivity to a wide range of aquatic stimuli; single odorants as well as a complex mixture. Although onset latency and duration of response cannot be strictly compared between cultured cells and that of intact non-cultured cells, due to the different odorant delivery systems required for each type of recording, odor response kinetics in each system are qualitatively similar (Michel, unpublished data). The cultured cells also display discrimination properties exemplary of olfactory neurons in as much as the magnitude of evoked current is concentration dependent and currents are not evoked by every stimuli that is presented. The percentage of total cells responding to a single presented odorant is greater than half; a percentage which increases as cells are sequentially presented with a variety of odorants or with a complex mixture. This degree of selectivity to odors would be expected if the substances were activating odor receptors and not receptors for some more generalized function, such as modulation of sensitivity. These data suggest that each cell is responding to specific odors and that the likelihood of applying the appropriate stimuli increases when cells are screened with a number of compounds; three to five as opposed to just one. I tentatively conclude that the "neuron-like" cells are not only olfactory, but that they retain their ability to respond to odor compounds selectively.

It is somewhat surprising that only poly-D-lysine provided a suitable substrate for neurite outgrowth of the olfactory neurons. Poly-D-lysine was found to be inferior to Con A or uncoated plastic (Falcon 3001) and inferior to uncoated Primaria dishes (Falcon 3801) or polyornithine for culturing chicken (Gonzales et al., 1985) and insect (Stengl and Hildebrand, 1990) olfactory receptor cells, respectively. Neonatal rat olfactory cultures maintained on poly-D-lysine also displayed poor plating efficiency (Ronnett et al., 1991). Krenz and Fischer (1988), found only 40 to 50% survival of crayfish stomatogastric ganglia neurons when plated onto poly-D-lysine. Graf and Cooke (1990), however, did find extensive neurite outgrowth of stomatogastric neurons from lobster or crab when plated onto poly-D-lysine, but they also found cell attachment to uncoated Primaria dishes (Falcon 3801). In my cultures, albeit from the same family (lobster), plastic or uncoated glass substrates prevented attachment of cells and resulted in cell death. One could argue that neurons from sensory organs may have similar substrate requirements. Little neurite outgrowth, however, is seen in retinal ganglion cell cultures plated onto poly-D-lysine, while extensive networking processes develop on substrates of laminin and fibroblasts (Drazba and Lemmon, 1990). While minimal process outgrowth was observed in neonatal rat olfactory neurons plated on poly-D-lysine, significant neurite outgrowth was obtained when laminin was applied to poly-ornithine treated slides (Ronnett et al., 1991). Although, in my cultures, laminin provided the highest initial plating density in terms of survival of total number of cells at 2 hr after plating, cells failed to adhere to this substrate and subsequently died. My observation that cells died within 36 hr if they did not adhere to an appropriate substrate and, as a population, extend neurites, is consistent with Cooke et al. (1989) findings that cultured crab or lobster peptidergic neurons had to adhere to a substrate for support and outgrowth. Thus, the optimal substratum for neuron survival and process outgrowth is not necessarily genus specific, may be dependent on type of nervous tissue, and may even vary among neurons cultured exclusively from different sensory organs.

My observation that supplementation 2 hr after plating yields even greater cell densities than if supplied immediately upon plating, suggests that an initial period of neuronal-substrate contact without FCS may be important, perhaps to prevent FCS-induced aggregate formation. This finding is in accordance with Sebben et al. (1990) who find that both the amount and timing of serum introduction are critical factors in long-term primary cultures. They report non-neuronal cell death during FCS deprivation; in their work, up to 72 hours after plating. Although, in my system, selective mortality of the non-neuronal cells could be achieved only after 24 hours, it was not necessary to achieve my goals with lobster antennular cultures. The larger non-neuronal cells only comprised about 5% of a given culture, did not proliferate to confluency to affect survival of the smaller, neuronal cell types, nor did their presence interfere with my electrophysiological measurements.

While clotted lobster serum (haemolymph) provided an excellent substrate in terms of survival and neurite extension, it was not conducive to electrical recordings. Serum and, presumably, proteins in the haemolymph substratum interfered with the formation of high resistant gigaohm seals required for patch-clamp recordings. In contrast to cells cultured with FCS, which could be rinsed with serum-free L15 media prior to recording to alleviate this difficulty, haemolymph could never be sufficiently washed from cell surfaces.

Many sensory neurons have the biological requirement for nerve growth factor (NGF) which regulates survival, development, and maintenance of these neurons in vertebrate systems (Johnson et al., 1986; Lindsay et al., 1990; and Paves et al., 1990). The fact that NGF-7s had no significant effect on longevity or neurite outgrowth could be explained by the fact that the cells were always serum supplemented. FCS has many undetermined neurotrophic factors which could sustain olfactory neurons in culture. Hence, any additional effect (increased longevity, neurite outgrowth, or electrical excitability) from NGF-7s might have been masked by factors contained in the FCS.

There are many conceivable reasons why cultures derived from tissues of recently captured animals survived up to twice as long as those derived from animals held in captivity for 8 weeks or more. The animals may not have been as healthy as those in their natural environment, even though food quality, water turnover, and cleanliness of aquaria were carefully monitored. Certainly, other researchers studying various aspects of olfaction (as well as those in my home laboratory) have discovered desensitization of neurons in catfish and salamander when animals were held for as little as 2 weeks (Caprio and Firestein, pers. comm.).

While the remaining tested parameters collectively improved longevity, individually, no single factor appeared pivotal for cell survival. The optimal osmolarities (965, 989, and 1082 mOsm) were congruous with the measured osmolarity (1079 mOsm) of haemolymph. The temperature which gave the greatest cell survival (24°C), was lower than the reported optimal environmental temperature for this species (29-30°C; Lellis and Russel, 1990). Since the above cited study was based largely on behavioral repertoire, frequency of molting, and growth rate; and the variation found between tested temperatures using these indices was minimal (95% to 99% survival within a temperature range of 25 to 29°C, respectively), the actual temperature optima for physiological processes in culture may not precisely coincide. The requirement for high humidity may have acted indirectly by preventing changes in osmolarity, although at less than saturated humidity evaporative losses were minimal. Measured osmolarity change in media over any one week in time was no more than 20-30 mOsm at 60-80% saturation. There appeared, then, to be a broad range of suboptimal conditions (Figure 2-2) which were suitable for cell maintenance but not for any apparent new growth (indexed by neurite extension).

In one of the two other reports in which dissociated crustacean neurons were cultured (Cooke et al., 1989; Graf and Cooke, 1990), outgrowth was observed in a simple medium of only physiological saline and glucose. Although both my work and theirs used cells from congeneric lobsters, the type of neurons cultured in each study differed. Secretory neurons from the X-organ-sinus gland (Cooke et al., 1989) may be able to use existing membrane from stored granules to actively synthesize proteins or regulate transport mechanisms for immediate neurite outgrowth. Lobster olfactory neurons, in contrast, while not requiring preconditioned media, had a strong dependence (among other noted factors) on neurotrophic factors supplied in serum or haemolymph. The regenerative nature of olfactory neurons, as primary receptor neurons, may require the strict presence of specific physiological factors for continual neurite outgrowth, and subsequent viability in culture. My findings appear to be more analogous to the other reported work on crustacean neuron cultures, that of Krentz et al. (1990) who also found serum supplementation (5-10% FCS) necessary for sustained growth.

Although appropriate target organs have been known to influence proliferation and direction of neurite outgrowth in culture (Coughlin, 1975; Pollack et al., 1981), this appears not to be the case for growing olfactory neurons of rat and chicken (Gonzales et al., 1985), or of the lobster, since lobster olfactory neuron cultures can be sustained without the presence of the lobes and preconditioned media had no affect on viability or neurite outgrowth. This finding is in contrast to cultured insect olfactory receptor neurons that have been reported to fail within 2d in the absence of conditioned media (from either non-neuronal antennal cells, extracellular fluid from antennae, or from the hormone 20-hydroxyecdysone) (Stengl and Hildebrand, 1990). It is possible that the insect olfactory neurons have more rigid culture requirements than that of rat, chicken, or lobster, since the latter systems were developed from differentiated adult cells and not embryonic cells, as was the case for the insect. In intact organ cultures or in large transplants of olfactory organs (Morrison and Monti Graziadei, 1983), however, appropriate targets may be influential in promoting olfactory neuron growth. Perhaps, the culture systems of the rat, chicken, and lobster may have not allowed sufficient cell-cell interaction for targets to be effective, since they were all plated at low density (range 3×10^5 to 1×10^6 cells/ml).

Two recent culture systems of rat olfactory receptor cells, one a continual clonal cell line from olfactory epithelium (Coon et al., 1989) and another of isolated olfactory neurons (Ronnett et al., 1991) show increased cAMP levels in response to odor stimulation, but being based on biochemistry and not electrophysiological recordings, did not give information on the odor specificity of single cells. The high survival, electrical excitability, and odor responsiveness that I find in cultures of single lobster olfactory receptor cells has also been reported in monolayer cultures of isolated rat olfactory tissue (Pixley and Pun, 1990). Identical to cultured lobster olfactory neurons, neurons from rat evoke a fast inward current, followed by an outward current, when depolarized to -30 mV. Upon application of odorant mixtures (single compounds were not tested), rat olfactory neurons produced only inward current under voltage-clamp conditions (Pixley and Pun, 1990). In contrast, cultured lobster olfactory neurons produced either outward or inward currents in response to odorant mixtures and to single compounds; a finding that presumably corresponds to the previously noted depolarizing and hyperpolarizing receptor potentials in lobster olfactory receptor cells *in situ* (McClintock and Ache, 1989b). This may imply a functional difference between lobster and rat olfactory cells in their ability to produce currents of both polarities, but it may also reflect the limited set of odors tested on the rat olfactory neurons. Odor-evoked decreases in action potential frequency that may reflect an underlying outward current have been reported in olfactory receptor cells in another vertebrate (Dionne, 1990).

Panulirus argus olfactory receptor neurons can now be considered among a small, yet growing number of culturable crustacean cells. More importantly, they join a phylogenetically diverse group of olfactory receptor neurons that can maintain odor sensitivity in culture. Since lobster olfactory receptor cells in culture are morphologically compact, it should be possible to obtain an effective space-clamp for recording odor-activated currents; drug introduction and incubation periods can be simplified and extended, respectively. Moreover, the cells are directly accessible for electrophysiological recordings. In light of these technical benefits, the cultured system should provide a useful model for future studies of olfactory transduction as exemplified in Chapters 3 to 7.

CHAPTER 3
ODOR SENSITIVITY IS NOT DEPENDENT ON PROCESS FORMATION

Introduction

The small size and thin, elongated morphology of olfactory receptor neurons (ORNs) was long an impediment to understanding olfactory transduction. The advent of patch-clamp recording ameliorated this situation and facilitated progress toward understanding olfactory transduction (reviews: Anholt, 1991; Firestein, 1991). Central to this effort has been the ability to dissociate ORNs from their surrounding epithelium in order to study them directly or in sustained primary culture. Dissociated ORNs are not only accessible for patching, they often assume a more compact form than their counterparts *in situ* that allows a reasonable space clamp and facilitates diffusion of membrane impermeant probes from the electrode to the site of transduction. While transduction is thought to occur in the cilia (outer dendrites, in invertebrates) of the ORNs (e.g., Kurahashi, 1989; Firestein et al., 1990; Lowe and Gold,

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1991), at least some elements of the transduction cascade are not confined to the cilia. Specifically, cAMP-gated cation channels that are the effectors in the transduction cascade in amphibian ORNs also occur on the dendrite and soma of the cells, although in lower densities than on the cilia (Firestein et al., 1991; Zufall et al., 1991a). Indeed, this variability in density was exploited to obtain favorable channel density for recording (ibid.).

Previously, in Chapter 2, preliminary evidence was reported that cultured lobster ORNs respond to odors independently of whether the cells had sprouted processes. This observation raises the interesting possibility that, *in vitro*, **all** elements of the transduction cascade may be expressed and inserted into the soma of ORNs prior to or independent of process formation. Given the ease of patching the soma compared to the extremely thin cilia (outer dendrites) and the ability to culture ORNs in vertebrates (Coon et al., 1989; Pixley and Pun, 1990; Calof and Chikaraishi, 1991; Ronnett et al., 1991) and other invertebrates (Stengl et al., 1989; Zufall et al., 1991b), such a phenomenon could be of general utility for studying olfactory transduction.

Without the normal polarity of the cell, however, it must be established that applied "odors" are activating what would otherwise be ciliary (dendritic) chemoreceptors. The need to establish the adequacy of odor stimuli is particularly important when studying ORNs from aquatic animals such as fish and lobsters. Adequate olfactory stimuli for many aquatic animals are the blood-born components of prey, compounds such as amino acids, amines and nucleotides (review: Carr, 1990). These types of compounds could be expected to activate cells as neurotransmitters or neuromodulators. In order to establish the utility of cultured lobster ORNs for analysis of transduction mechanisms, I will in this chapter, provide functional evidence that cultured lobster ORNs with no or varying numbers of processes are morphologies of the same type of cell and that the odor-evoked properties of the cultured cells reflect those of lobster ORNs *in situ*.

Methods

Tissue Culture

The distinct clusters of the ORNs were dissected from the aesthetasc (olfactory) sensilla on the lateral antennular filament (olfactory organ) of adult specimens of the Caribbean spiny lobster, *Panulirus argus*. The clusters were enzymatically dissociated, and the resulting cells sustained in primary culture as described previously (Chapter 2). Briefly, the isolated clusters were incubated for 50 min at 80 rpm on an orbital shaker in 0.2 micron filter-sterilized solution of 2.5 mg papain and 12 mg L-cysteine in 10 ml *Panulirus* saline (PS) containing 1% penicillin, streptomycin sulfate, and amphotericin B

(Gibco). Proteolytic digestion was stopped by replacing the enzyme solution with low glucose L-15 media supplemented with L-glutamine, dextrose, fetal calf serum, and BME vitamins. Cells were immediately plated on poly-d-lysine-coated glass coverslips. Cells were maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) at 24°C. Neurite outgrowth in individual cells was recorded on a TL Panasonic 6050 time-lapse video cassette recorder. Images were later captured and subsequently analyzed using Image 1 analysis software.

Electrophysiology

Voltage- and odor-activated currents were recorded in the whole-cell configuration with an integrating patch-clamp amplifier (Dagan 3900). The analog signal was filtered at 5 kHz and digitally sampled every 4 msec. Data acquisition and subsequent storage and analysis of the digitized records were done with pCLAMP software (Axon Instruments). Cells were viewed at 40X magnification with Hoffman optics. Patch electrodes, pulled from 1.8 mm O.D. borosilicate glass, were fire polished to a tip diameter of approximately 1.0 μm (Bubble number 4.8; Mittman et al., 1987). High resistance seals (8.0 to 14 GΩ) were formed by applying gentle suction to the lumen of the pipette upon contact with the cell. In all experiments, cells were voltage-clamped at a holding membrane potential of -60 mV. Membrane resistance changes were determined by injecting current sufficient to elicit

30 mV, 300ms hyperpolarizing voltage steps into the cells from the holding potential. Each cell was photographed to allow correlation of soma size, length of process, and cell morphology with physiological properties.

Odor Stimulation

Odors were "spritzed" on the cells for 120 msec from a 7 barrel (6 barrels used) glass micropipette (Frederick haer) coupled to a pressurized valve system (Picospritzer, General Valve). In most trials, one randomly selected barrel was filled with fluorescein to permit positioning of the tip of the pipette relative to the cell and to assure that the delivered odorant completely surrounded the cell and its associated processes. The magnitude of the response to odors was determined to be independent of which of the six barrels contained the odor. Dilution of the odor between the pipette and the cell surface, an average distance of two cell diameters, was estimated to be approximately 9%, based on the calculated K^+ permeability method of Firestein and Werblin (1989). Odor concentrations are reported as the pipette concentration and are not corrected for this dilution.

The odors used were solutions of: (1) an equimolar mixture that included (10^{-3}M): betaine, glycine, lactic acid, taurine, and trimethylamine oxide, referred to as S-1; (2) an aqueous extract of TetraMarin, a commercially available fish food, prepared as described earlier

(Schmiedel-Jakob et al., 1990) and diluted 1000 fold, referred to as TET; and (3) single substances known to be effective odors for the lobster, which included (10^{-3}M): adenosine monophosphate (AMP), arginine, ascorbic acid, betaine, cysteine, glycine, histamine, proline, taurine, and trimethylamine oxide (TMAO). All odorant solutions were prepared daily in modified L15 media and applied at the stated concentration, unless otherwise noted.

The number of different odors that stimulated a given cell (the response spectrum) was quantified using the breadth of responsiveness metric of Smith and Travers (1979). Here, the breadth of responsiveness (H) is defined as

$$H = -K \times \sum_{i=0}^{n} p_i \mathrm{Log} p_i$$

where K = a proportionality constant, n = the number of odors tested, $p_i = |pA|$ the absolute current (pA) elicited from the nth odor and expressed as a proportion of total pA elicited from all odors.

Solutions

Panulirus saline (PS) consisted of (in mM) 458 NaCl, 13.4 KCL, 9.8 $MgCl_2$, 13.6 $CaCl_2$, 13.6 Na_2SO_4, 3 HEPES, and 2 glucose; pH 7.4. Modified L15 Media consisted of 50 ml Liebowitz L15 Stock, 50 ml of 1.6X normal concentration of PS, 0.6g dextrose, 0.026 g L-glutamine, and 0.01% gentamicin. The patch electrode solution consisted of (in mM)

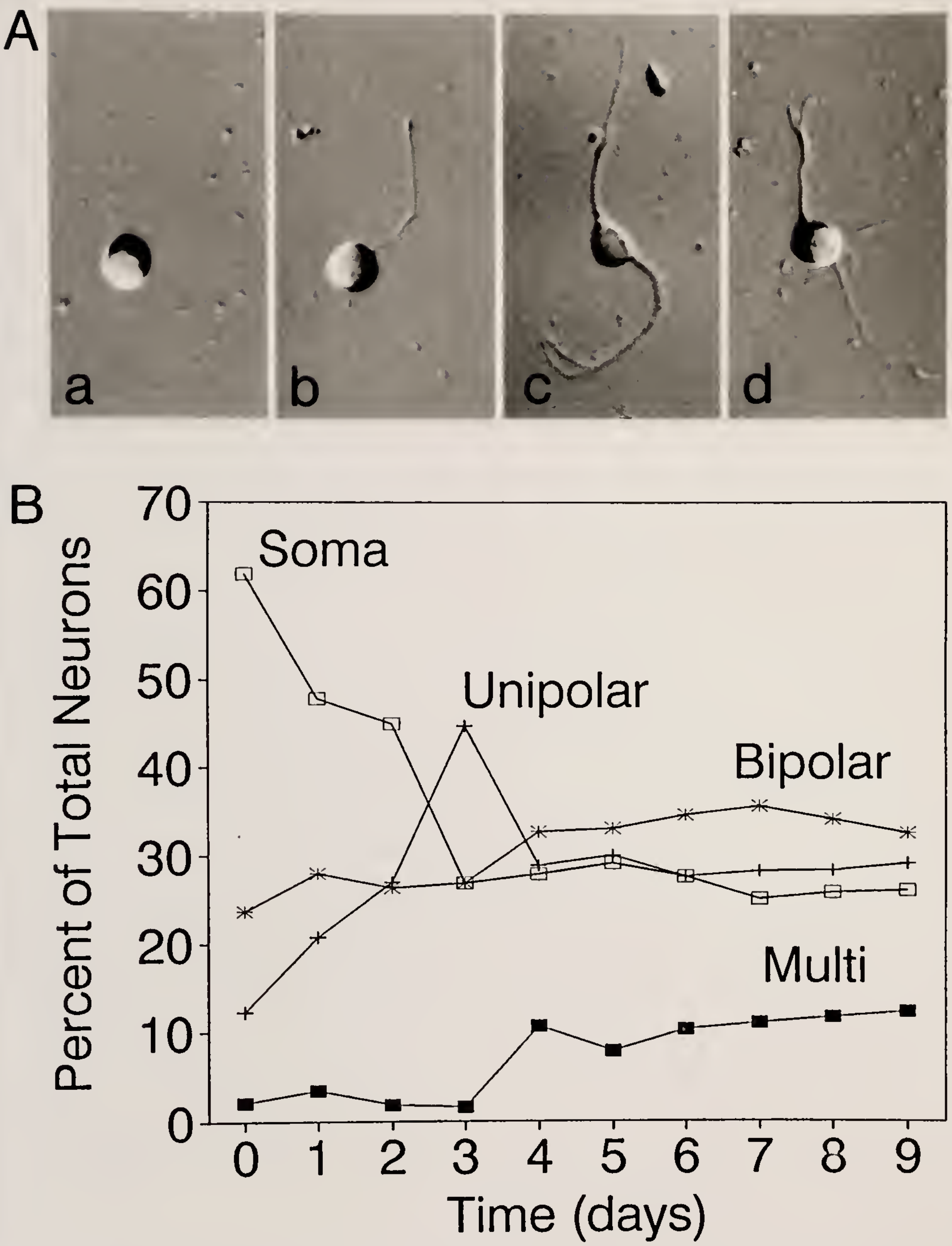
30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 180 K-acetate, and 696 glucose; pH 7.0. All salts were obtained from Sigma.

Results

Morphology: Neurite Outgrowth

The cultures consisted largely of the five morphological types of small (8-16 μm diameter soma) "neuron-like" cells, described previously (Fadool et al., 1991b): (1) soma only, (2) soma with bud, (3) unipolar, (4) bipolar, or (5) multiprocess, four of which were used in the present chapter (Figure 3-1A). The processes ranged from 3 to 160 μm long. Each of the four morphological types were present as early as two hours post-plating. Initially, the predominant form was "soma only", but the proportion of each morphological type changed over time; the relative proportion of cells lacking processes decreased, with a concomitant increase in the proportion of cells with processes (Figure 3-1B). To distinguish whether the change in the relative proportion of the morphological types reflected selective loss of cells lacking processes or process proliferation, or both, 218 "soma only" cells were followed individually with digital time-lapse imaging for three consecutive days starting 2 hr after plating. Forty-two (20%) of the cells died within the observation period, indicating that selective loss of "soma only" contributed to the change in the relative proportion of the cell types. While many of the 176 cells that persisted

Figure 3-1. A) Light micrographs of four morphs of cultured lobster ORNs observed under Hoffman modulation contrast optics. (a) soma only, (b) unipolar, (c) bipolar, (d) multipolar. Magnification 780X. B) Changes in the proportion of the four morphs over 9 days in culture. Each data point represents the incidence of that morph in 10 random fields of view, expressed as a percentage of all four types.

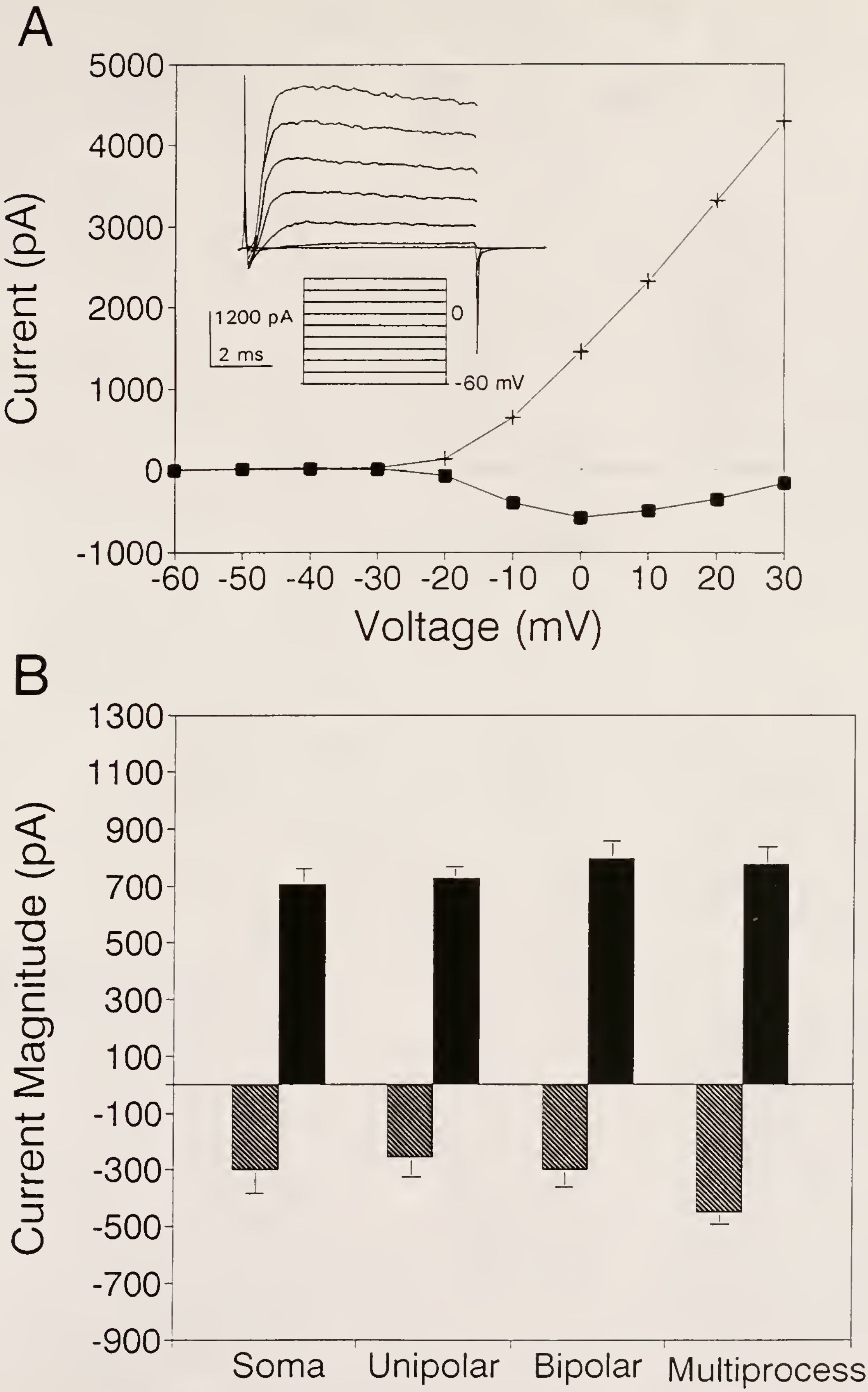

A
a
b
c
d
B
Soma
Unipolar
Bipolar
Multi
Percent of Total Neurons
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
Time (days)

failed to sprout processes, 61 of the cells sprouted processes throughout the observation period, becoming uni-, bi- and, eventually, multipolar. The latter finding supports my contention that the four morphological types of "neuron-like" cells were morphs of a single type of cell.

Physiology: Electrical Properties

That the four types of "neuron-like" cells were morphs of a single type of cell was supported by the finding that 268 cells tested for their current-voltage relationship had similar voltage-activated properties. The total membrane current evoked in a typical cell by depolarizing voltage-steps consisted of a transient inward current ($\leq$ 570 μsec duration) that activated around -30 mV, followed by a much larger, prolonged outward current that activated around -20 mV and persisted with little decay throughout the 15 msec duration of the pulse (Figure 3-2A). The magnitude of the inward and outward currents was independent of the number of processes on the cell (ANOVA, $p = 0.47$ - inward currents and $p = 0.71$ - outward currents) (Figure 3-2B). A subsample of 12 cells, including at least one cell of each morphological type, had a mean input resistance (R_N) at rest of 1.1 ± 0.2 GΩ and a membrane time constant (τ_0) of 67.3 ± 11.3 msec. No detectable equalizing time constant could be measured in any of the morphs, including morphs with multiple processes.

Figure 3-2. Total voltage-activated currents of cultured lobster ORNs.
A) Representative current-voltage relationship of one cell. ■ = inward current, + = outward current. Inset: Macroscopic currents (upper traces) evoked by depolarizing voltage-steps (lower traces) when the cell was held at -60 mV and stepped to +30 mV in 10 mV episodes. Records are not leak subtracted. B) Plot of the maximum amplitude (mean ± SEM) of the inward (striped bars) and outward (solid bars) currents of 268 cells grouped according to number of processes (n=68 soma only, 76 unipolar, 69 bipolar, 55 multiprocess).


A
Current (pA)
5000
4000
3000
2000
1000
0
-1000
1200 pA
2 ms
0
-60 mV
-60
-50
-40
-30
-20
-10
0
10
20
30
Voltage (mV)
B
Current Magnitude (pA)
1300
1100
900
700
500
300
100
-100
-300
-500
-700
-900
Soma
Unipolar
Bipolar
Multiprocess

Physiology: Response to Odors

472 cells (including cells tested above for their electrical properties) were tested for their ability to generate a current in response to stimulation with one to five different odors. The odor arrays usually, but not always, included the complex mixture, TET. Sixty-four percent of the cells tested responded to at least one odor. This percentage increased to 89% when the cells could be tested with at least three different odors (n=182). Odors evoked a transient current that rose to a maximum over several hundred msec and subsequently declined more slowly to rest (Figure 3-3). The current could be of either polarity, depending on the cell and the odor tested, and different odors could evoke currents of opposite polarity in the same cell (Figure 3-3). In cells that could be tested with at least three different odors (n=182), the odors that were tested evoked only inward currents in 48 cells (26%), only outward currents in 58 cells (32%) and currents of both polarities in 56 (31%) cells. The remaining 11% of the cells did not respond to any of the odors tested.

Odor-evoked currents of both polarities were associated with an increase in membrane conductance, as indicated by a decrease in input resistance, when hyperpolarizing voltage-steps were injected into the cells prior to and during odor-stimulation (Figure 3-4). The input resistance (R_N) decreased significantly under odor stimulation from a mean

Figure 3-3. Whole-cell, voltage-clamp recording from a cultured lobster ORN in response to spritzing the cell (arrow) with 10^{-3} M proline (top trace), 10^{-3} M taurine (second trace), TET (third trace) and culture medium only (bottom trace). Holding potential, -60 mV for all traces.

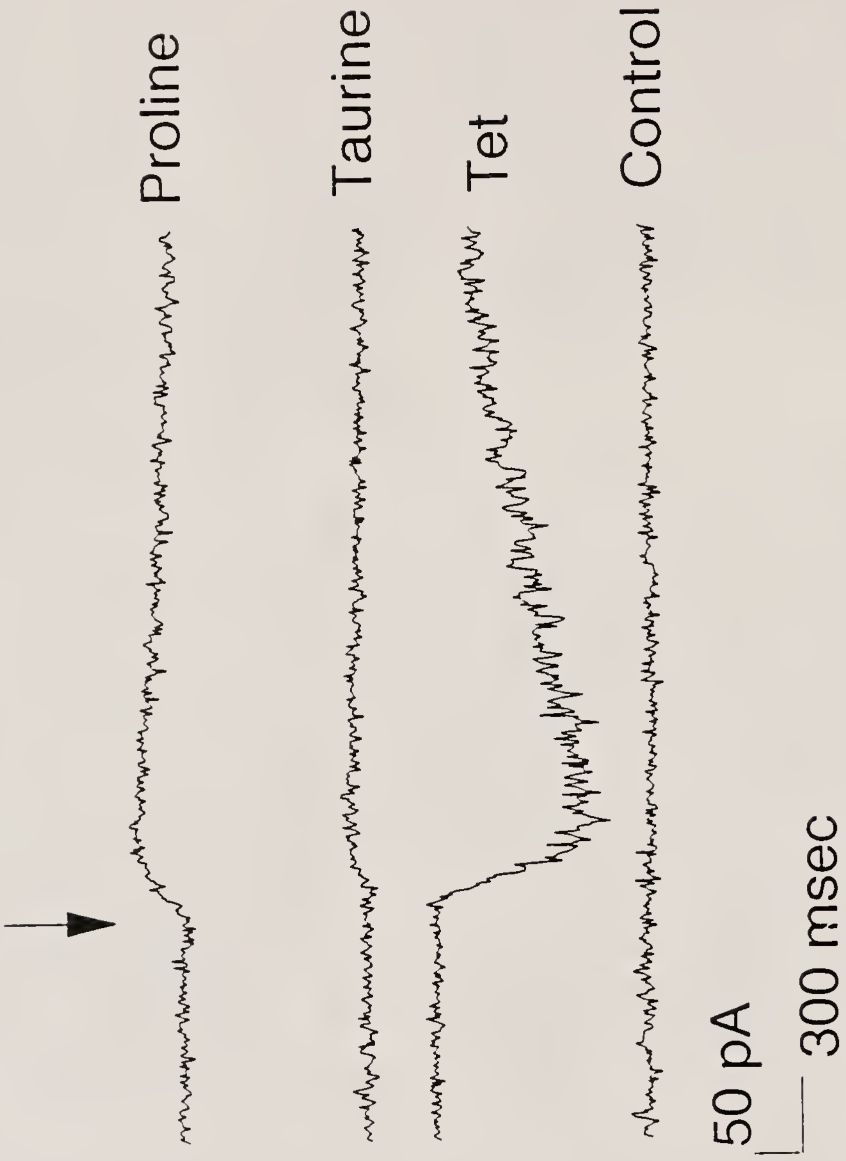
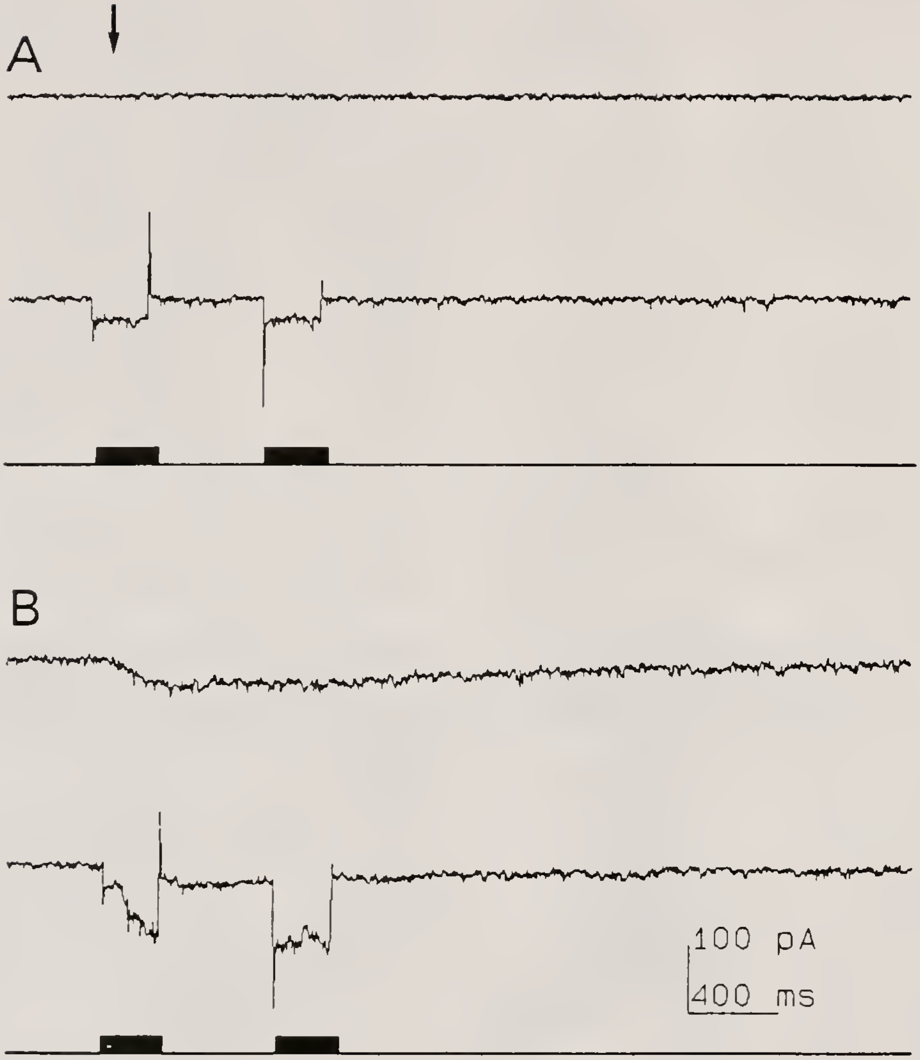

Proline
Taurine
Tet
Control
50 pA
300 msec

Figure 3-4. Whole-cell, voltage-clamp recordings from a cultured lobster ORN showing increased conductance associated with the odor-stimulated inward current. A) Neither current (upper trace) nor change in conductance (lower trace) was evoked by stimulating (arrow) with culture media control. B) Inward current (upper trace) associated with an increase in conductance (lower trace) was evoked by stimulating with TET. Holding potential, -60 mV. Solid bars denote 30 mV, 300 msec hyperpolarizing voltage pulses used to monitor membrane conductance.


A
B
100 pA
400 ms

of 1.1 ± 0.1 to 0.7 ± 0.1 GΩ for the inward current (n=8) and from a mean of 1.3 ± 0.6 to 0.7 ± 0.1 GΩ for the outward current (n=4) (paired t-test, $p < 0.05$). Concomitantly, the membrane time constant (τ_o), decreased significantly during odor stimulation from 59.6 ± 12.6 to 21.6 ± 4.1 msec for the inward current (n=14) and from 82.5 ± 20.7 to 34.6 ± 9.5 msec for the outward current (n=7)(paired t-test, $p < 0.05$).

The latency to the onset of the odor-evoked currents, measured from the activation of the spritzer, ranged from <20 msec to > 1 sec, but typically was <100 msec (Figure 3-5). Overall, the mean latency to onset for the inward current, 186.2 ± 31.3 msec (n=100), was significantly longer than that for the outward current, 81.0 ± 10.6 msec (n=121) (t′ Statistic, $p < 0.05$). To determine if this difference was possibly driven by between cell variation, I performed a paired comparison of the latency in 19 cells that supported odor-evoked currents of both polarities. The mean latency in these cells for the inward current was not significantly longer than that for the outward current (paired t-test, $p < 0.05$).

The peak amplitude of odor-evoked currents of both polarities increased with the concentration of the odor and saturated over 3-4 orders of magnitude (Figure 3-6). The mean slope of the concentration-response function in the steepest region of the curve was 8.3 ± 1.7 pA/decade (n=3) for the inward current and 1.3 ± 0.2 pA/decade (n=7) for the

Figure 3-5. Plot of the distribution of the latency of odor-evoked currents in cultured lobster ORNs. Striped bars, inward currents (n=100); solid bars, outward currents (n=121). Arrows denote the mean latency to onset of the inward (open arrow) and outward (filled arrow) currents, respectively.

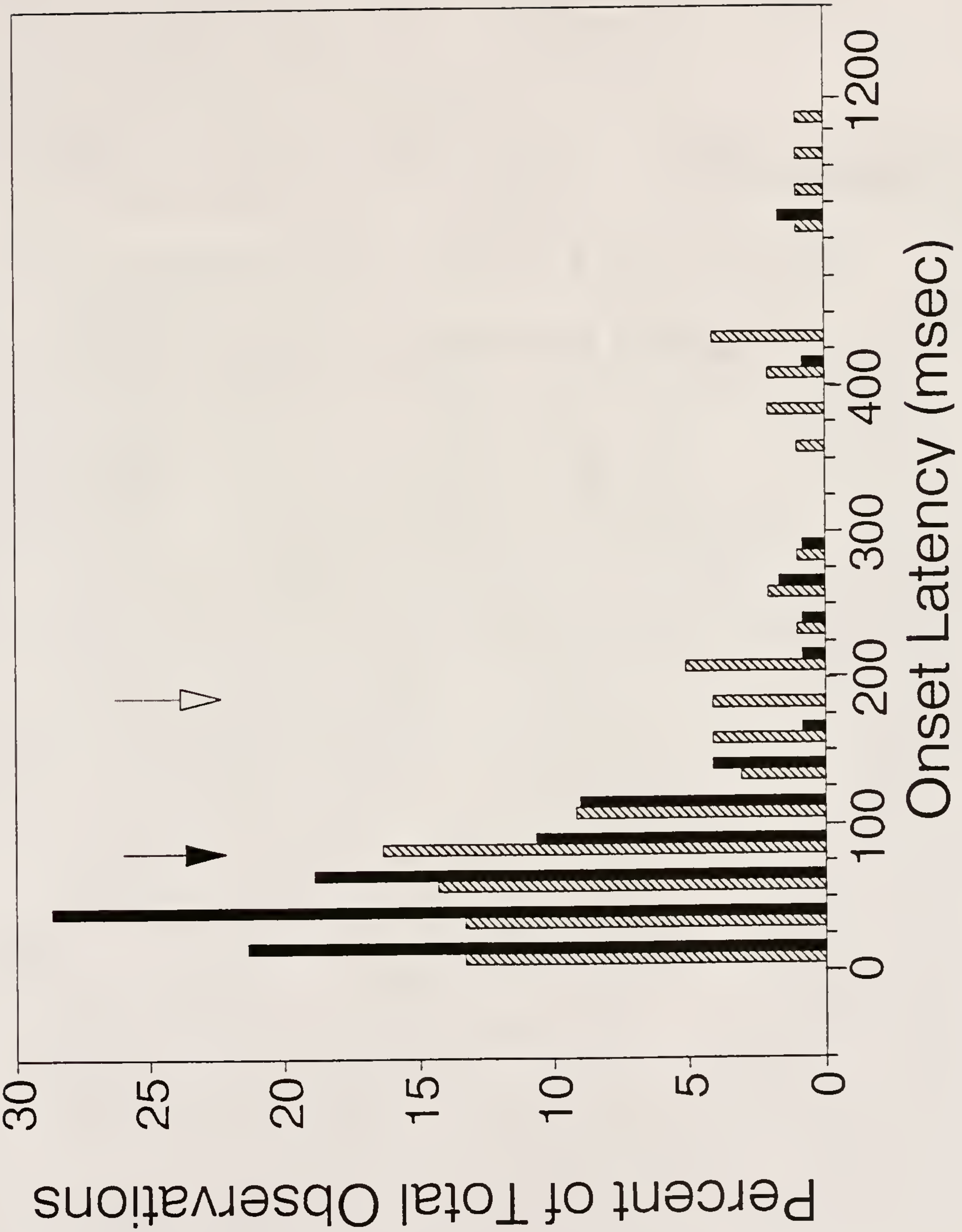
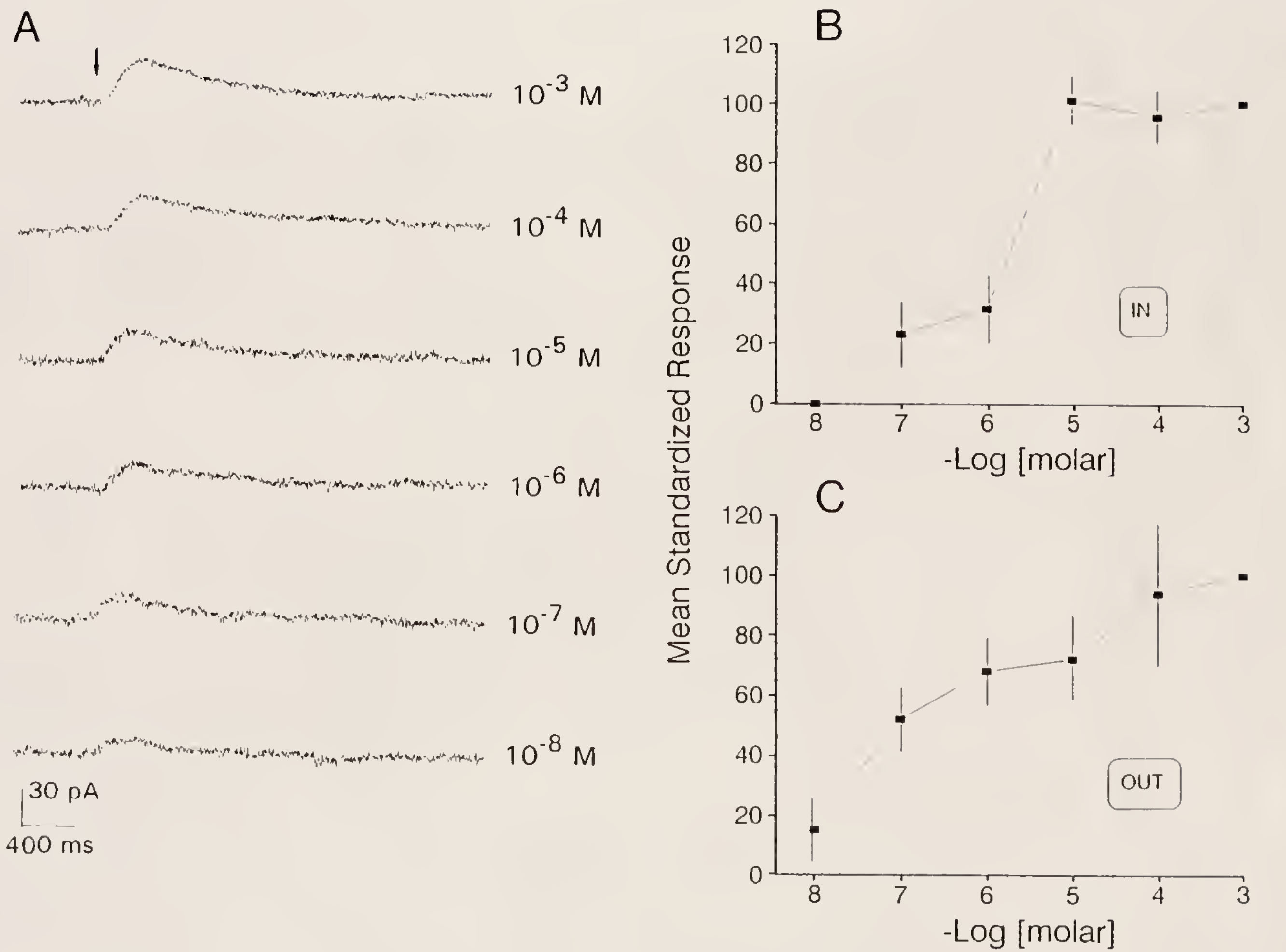

Percent of Total Observations
0
5
10
15
20
25
30
Onset Latency (msec)
0
100
200
300
400
1200

Figure 3-6. Dose response characteristics of cultured lobster ORNs. A) Whole-cell, voltage-clamp recordings of outward currents in a cultured lobster ORN evoked by stimulating the cell (arrow) with decreasing concentrations of proline. Holding potential, -60 mV. B) Plot of the peak inward current (X ± SEM) elicited in three cells by taurine at the concentrations shown. C) Plot of the peak outward current (X ± SEM) elicited in three other cells by proline at the concentrations shown. Current magnitudes in B and C are normalized to that evoked by 10^{-3} M odor.


A
10-3 M
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
30 pA
400 ms
B
C
Mean Standardized Response
120
100
80
60
40
20
0
8
7
6
5
4
3
-Log [molar]
IN
OUT

outward current. Thresholds were at least 10^{-8} M, the lowest concentration tested, for currents of either polarity.

The peak amplitude of currents of both polarities evoked by a single concentration (10^{-3}M) of odor ranged, typically, from 5-85 pA (Figure 3-7A). The average magnitude of the inward current (39.2 ± 3.0 pA) was significantly greater than that of the outward current (20.1 ± 1.4 pA) measured across all cells and odors (n=386; t′ Statistic, $p \leq 0.05$). The polarity and the magnitude of the currents evoked by odors were independent of the cell morphology for six different odors. The results for two of the six odors, proline (n=111 cells) and taurine (n=102 cells), are shown in Figure 3-7B,C. The peak amplitude of the odor-evoked currents of either polarity was also independent of the length of the process in cells bearing processes (n=55, correlation analysis, r > 3.86) (Figure 3-8A) and the size of the soma in cells lacking processes (n=60, correlation analysis, r > 3.86) (Figure 3-8B).

Single odors activated from 14 to 77 percent of the cells (Table 3-1). The stimulatory effectiveness was: betaine > histamine > glycine > proline > taurine > AMP > TMAO > ascorbate > arginine > cysteine. An equimolar mixture of five compounds (S-1: betaine, taurine, glycine, TMAO, lactate) ranked intermediate in stimulatory effectiveness. A complex mixture (TET) stimulated more of

Figure 3-7. Plots of the peak amplitude of odor-evoked currents in cultured lobster ORNs as a function of cell morphology. A) Distribution of the peak amplitude of the currents evoked in 386 cells by single odors tested at a common concentration (10^{-3} M). Striped bars, inward currents; solid bars, outward currents. Arrows denote the mean amplitude of the inward (open arrow) and outward (filled arrow) currents. B) Plots of the peak amplitude of currents of both polarities evoked by stimulation with 10^{-3} M proline (n=111) and 10^{-3} M taurine (n=102) grouped according to morphology. Inward currents are denoted as negative, outward as positive.

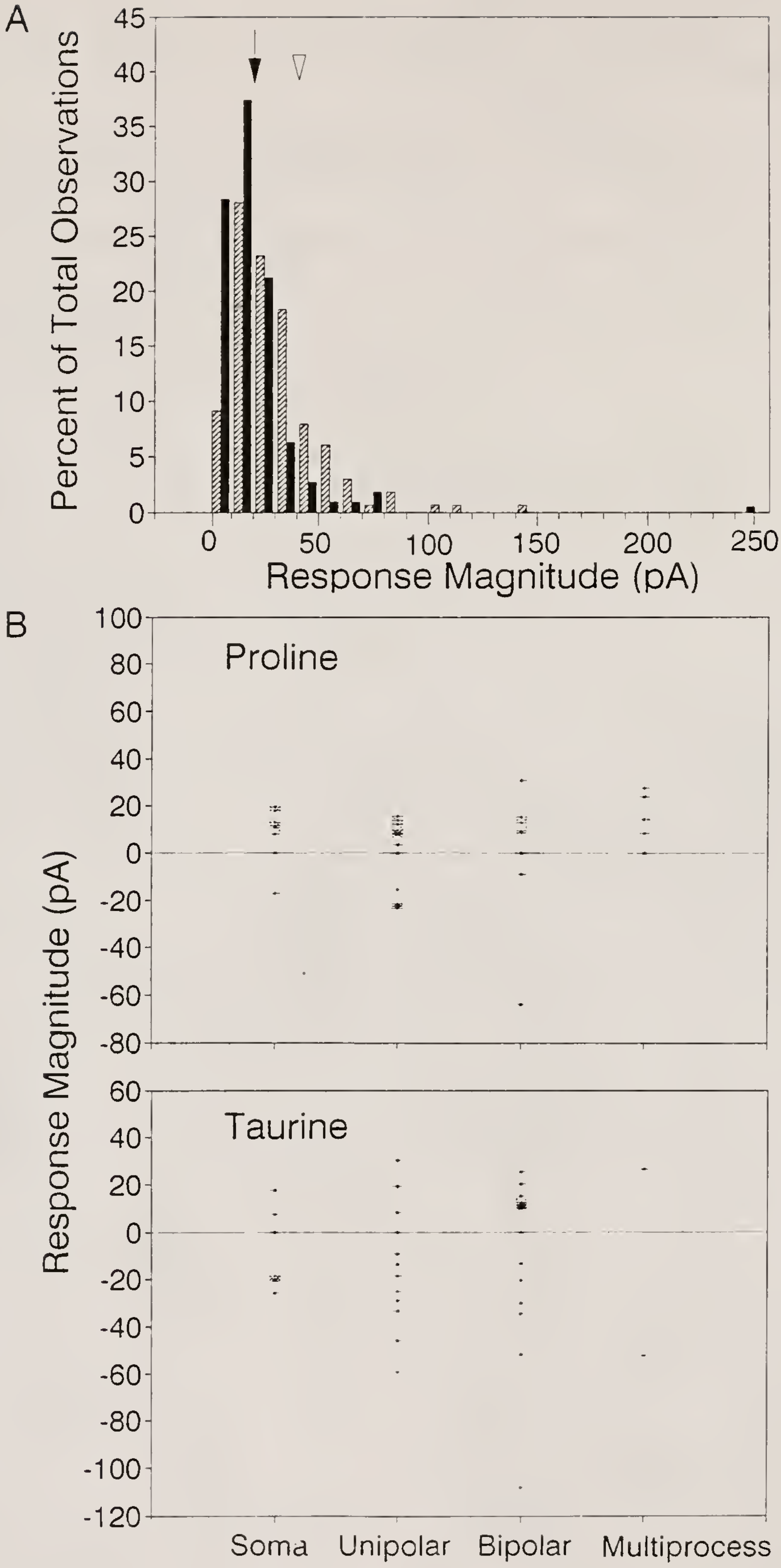

A
Percent of Total Observations
45
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
Response Magnitude (pA)
B
Proline
100
80
60
40
20
0
-20
-40
-60
-80
Taurine
60
40
20
0
-20
-40
-60
-80
-100
-120
Response Magnitude (pA)
Soma
Unipolar
Bipolar
Multiprocess

Figure 3-8. Plots of the peak amplitude of odor-evoked currents in cultured lobster ORNs. A) Amplitude as a function of length of the longest process (if more than one) for 55 cells B) Amplitude as a function of diameter of the soma in 60 cells lacking processes. The data set combines currents evoked by 10^{-3} M proline, arginine, taurine, TMAO and betaine as odors (1 cell, 1 odor). Inward currents in both plots are denoted as negative, outward as positive.

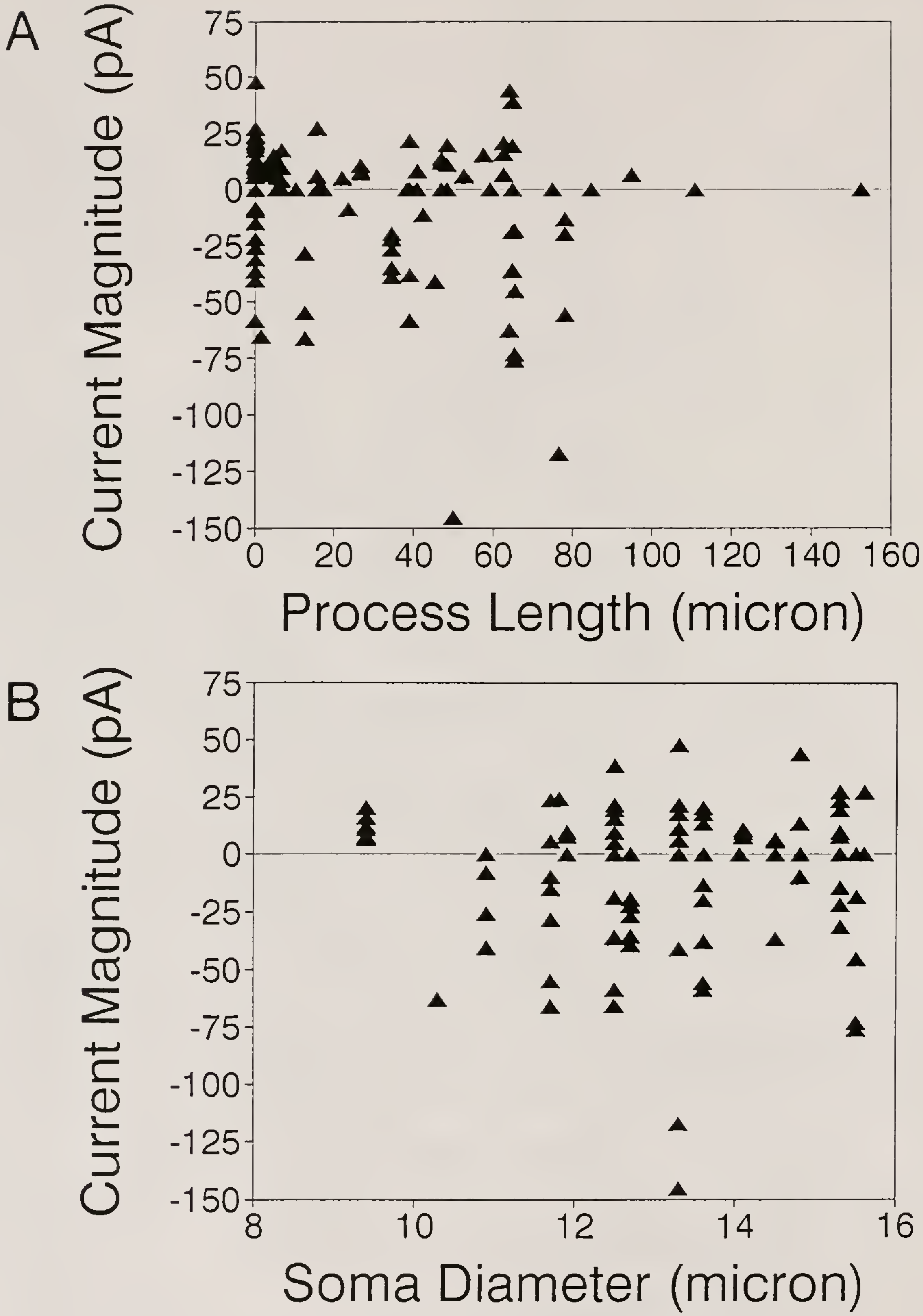

A
Current Magnitude (pA)
75
50
25
0
-25
-50
-75
-100
-125
-150
0
20
40
60
80
100
120
140
160
Process Length (micron)
B
Current Magnitude (pA)
75
50
25
0
-25
-50
-75
-100
-125
-150
8
10
12
14
16
Soma Diameter (micron)

Table 3-1. The percentage of cultured lobster olfactory receptor cells responding to individual odors, a mixture of 5 odors, and a complex odor.

Compound	Conc [mM]	# Cells Tested	# Cells Responding		% Responding to Odor
			Inward	Outward	
Control	-	237	0	0	0
Betaine	1	13	0	10	77
Histamine	1	4	1	2	75
Proline	1	68	13	30	63
Taurine	1	58	24	12	62
Glycine	1	14	5	5	57
AMP	1	10	1	4	50
S-1 Mix	5	16	4	3	44
TMAO	1	34	7	7	41
Ascorbate	1	8	0	2	25
Arginine	1	17	3	1	24
Cysteine	1	14	0	2	14
Proline	100	54	9	25	63
Taurine	100	52	20	7	52
Arginine	100	21	2	8	48
TET	0.1*	4	3	1	100
TET	0.001*	52	19	12	60

* = relative concentration of stock solution where 0.1 = 10X dilution of Tetramarin and 0.001 = 1000X dilution of Tetramarin; TMAO = trimethylamine-oxide; S-1 Mix = betaine, TMAO, glycine, taurine, lactate; Control = Liebowitz L15 Media; AMP = adenosine monophosphate.

the cells than did any single compound or the 5 component mixture, but this could be accounted for by concentration since the concentration of TET relative to that of the pure compounds is unknown. Culture medium itself, tested as a control, stimulated none of the cells tested, even though it contained some of the same amino acids as above as well as L-glutamine. Some odors selectively activated either inward or outward currents (Figure 3-9). Taurine elicited inward currents twice as frequently as outward currents, while proline showed the opposite tendency. Betaine and ascorbic acid elicited strictly outward currents, although the number of cells tested in each of these instances was not large.

In a separate experiment, 53 cells that were determined to be odor responsive by their ability to respond to TET were presented sequentially with the same five odors in order to determine their response spectra or "tuning". The cells varied in the number of odors they responded to, as well as the magnitude and the polarity of the response to any one odor (Figure 3-10A). The mean breadth of responsiveness for the sample population was $H = 0.31 \pm 0.04$ (Figure 3-10B). Repeating this analysis using fewer odors or the same number of different odors yielded measures of H between 0.2 and 0.3 (data not shown).

Figure 3-9. Plot of the percentage of cultured lobster ORNs that respond to various odors with inward and outward currents. All odors tested at 10^{-3} M, with the exception of TET, which was tested at 1000-fold dilution of stock. TMAO, trimethylamine-oxide. AMP, adenosine monophosphate.

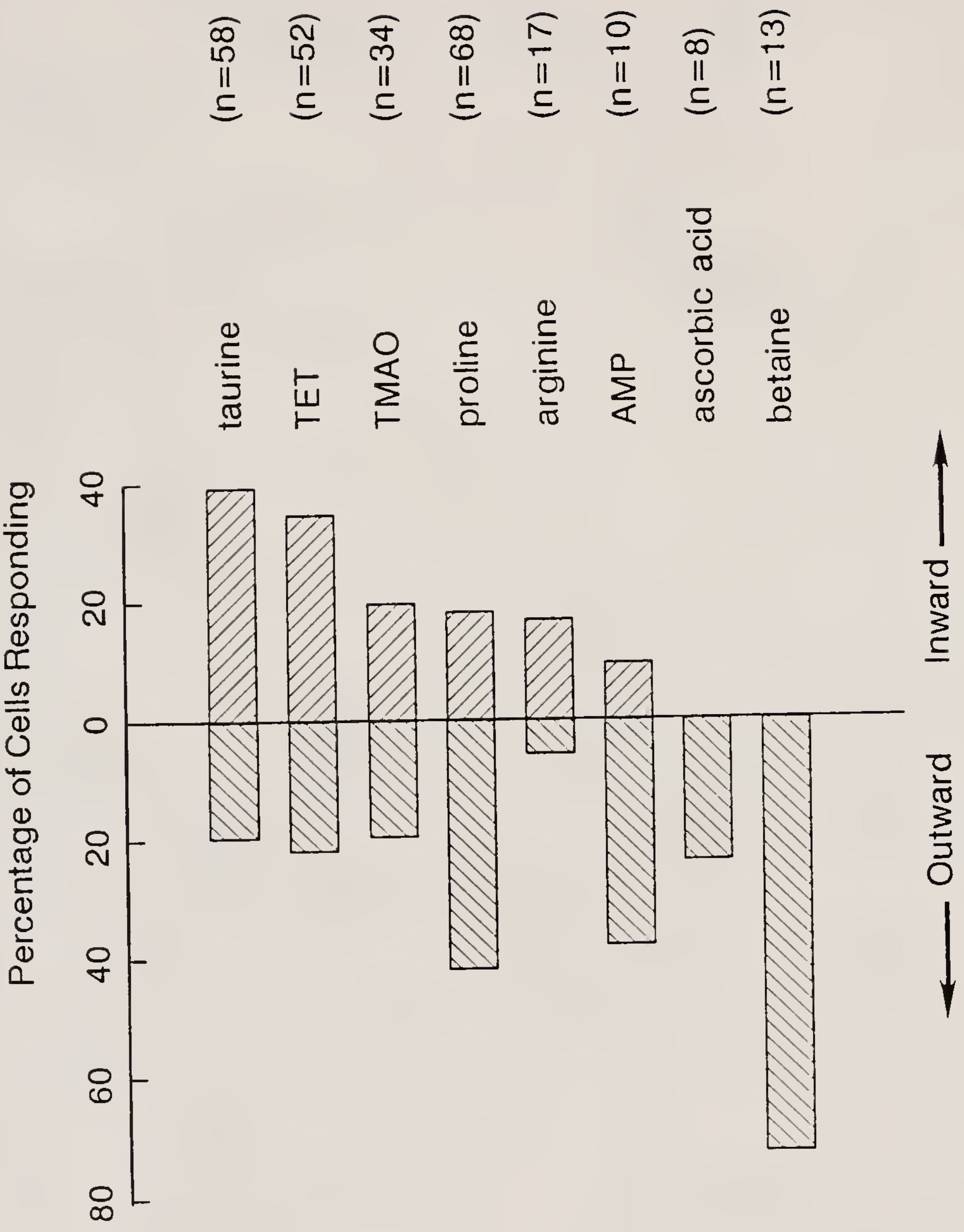
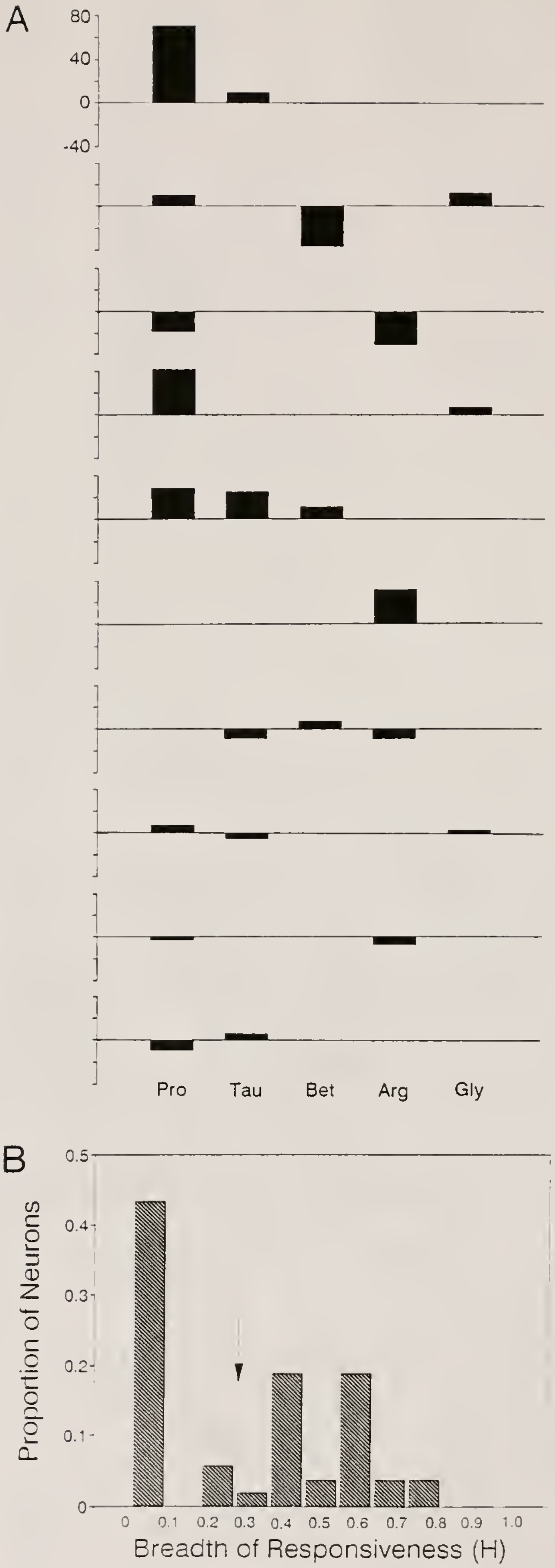

Percentage of Cells Responding
80
60
40
20
0
20
40
taurine (n=58)
TET (n=52)
TMAO (n=34)
proline (n=68)
arginine (n=17)
AMP (n=10)
ascorbic acid (n=8)
betaine (n=13)
Outward
Inward

Figure 3-10. Degree of odor tuning in cultured lobster ORNs. A) Graph of the response profiles of 10 cultured lobster ORNs to 5 odors tested at 10^{-3} M. Each horizontal line depicts the responses of a different cell; the height and direction of the bars on each line indicate the amplitude and the polarity, respectively, of the odor-evoked current (pA). Outward currents are denoted as positive (upward bars); inward currents as negative (downward bars). B) Plot of the breadth of responsiveness of 53 cells. Arrow denotes the mean H value.


A
80
40
0
-40
Pro
Tau
Bet
Arg
Gly
B
0.5
0.4
0.3
0.2
0.1
0
Proportion of Neurons
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Breadth of Responsiveness (H)

Discussion

The fact that the cells could be observed to sprout processes in culture, together with their common active electrical properties, strengthens the contention I proposed in the previous chapter that the various types of "neuron-like" cells in the culture are morphs of one type of cell, the ORN. This finding is consistent with the highly enriched source of the cultured cells; the somata were harvested from the lumen of the olfactory organ, which is literally filled with grape-like clusters of the somata of the ORNs (Grünert and Ache, 1988).

Although most neurons, as well as non-neuronal cells, are chemically irritable (e.g., Lerner et al., 1990), it is reasonable to interpret the responses of the cultured ORNs as olfactory. Most importantly, the cultured ORNs showed a diversity of response profiles (Figure 3-10). Postsynaptic or neuromodulatory receptors, in contrast, would be expected to be much more homogeneously distributed across the population of cells and show either the same or, if multiple receptors, a restricted set of response profiles across cells. For example, a histamine-gated chloride channel implicated in modulating the output of lobster ORNs *in situ* occurs on the soma of more than 95% of the cells (McClintock and Ache, 1989a). Second, the average breadth of responsiveness of the cultured ORNs (H = 0.31), approximated that of lobster ORNs determined *in situ* with a similar, but not identical, array of compounds ($H = 0.2$, Derby et al., 1984) and did not differ significantly from that of lobster ORNs determined *in situ* with the same array of compounds ($H = 0.35$, Student's t-test, $p < 0.05$, pooled SD - M. Wachowiak, unpublished data). Third, like in their counterparts *in situ*, odors evoked both inward and outward currents in the cultured ORNs, and different compounds could evoke currents of opposite polarity in the same cell (McClintock and Ache, 1989b). Odors that tended to frequently elicit currents of one polarity in the cultured cells, e.g., proline and outward currents, also did so in lobster ORNs *in situ* (Schmiedel-Jakob et al., 1990; Michel et al., 1991). That currents of opposite polarity were associated with an increase in membrane permeability argues, as occurs *in situ* (ibid.), that the odors were not up and down-regulating a single conductance but, rather, were regulating two distinct conductances through two parallel transduction pathways. Last, the threshold and dynamic range of the cultured ORNs matched that of lobster ORNs *in situ* to the same or similar types of odor molecules (Schmiedel-Jakob et al., 1989; W.C. Michel, unpublished data). I conclude that most, and possibly all, of the chemosensitivity evoked by cultured lobster ORNs is sensory, i.e., is mediated by the same receptors and transduction pathways found in the outer dendrites of lobster ORNs *in situ*, and is not the result of activating synaptic, neuromodulatory or other receptors that may occur on the soma of lobster ORNs.

The inability of the culture medium to elicit responses from the cells when applied as a control stimulus, even though it contains amino acids shown to be odors (cysteine, glycine, arginine) and others (e.g., glutamine) that are potential odors, is not necessarily surprising. The cells were maintained and tested in the same medium and would be expected to adapt to continuous background stimulation, as do their counterparts *in vivo* (Borroni and Atema, 1988). However, adaptation to continuous background stimulation does shift the apparent threshold of the cells *in vivo* so that recording in medium might have lowered the overall sensitivity to those test stimuli in the medium (cysteine, glycine, arginine). Indeed, the smallest percentage of cells tested responded to cysteine and arginine; but, in contrast, glycine was among the more effective odors tested (Table 3-1). The remaining test odors, including those used for threshold determininations, were not components of the culture medium.

The overall magnitudes of the current evoked by odors in cultured cells could differ appreciably from those *in situ* due to possible differences in the number of channels expressed in culture. It is interesting, therefore, that the average magnitudes of the odor-evoked currents in the cultured ORNs (39.2 ± 3.0 pA, inward; 20.1 ± 1.4 pA outward) were within an order of magnitude of those evoked in lobster ORNs *in situ*. A 10-fold greater concentration of TET than that used in the present study elicited an average inward current of 25.4 ± 5.1 pA, while the same concentration of proline as that used in the present study elicited an average outward current of 4.5 ± 0.6 pA in lobster ORNs *in situ* - (Michel and Ache, in press). Odor-evoked currents of dendritic origin *in situ* would be electrotonically attenuated in the soma (where they were recorded), so the actual magnitude of the current in the dendrite would presumably be closer to that found in the cultured cells. My results are consistent with the possibility that the cultured cells express a full complement of odor-activated channels.

The average amplitude of the odor-evoked inward currents in lobster ORNs is an order of magnitude smaller than those reported for salamander or rat ORNs (Kurahashi, 1989; Pixley and Pun, 1990; Firestein et al., 1991; Lowe and Gold, 1991), but is consistent with the still limited understanding of unitary currents that may underlie the macroscopic current. Inositol 1,4,5-trisphosphate (IP_3), the suspected excitatory second messenger, activated 1-3 channels in most (49/56) cell-free patches taken from the plasma membrane of the cultured cells (Fadool and Ache, 1992a). On the assumption that these IP_3-activated channels are transductory, there are an estimated 330 to 1990 channels per cell, given a pipette tip diameter of 0.5 - 1.0 μm and a total cell surface area of 380 μm^2 (Fadool et al., 1991b); or 0.9 - 5.3 channels/ μm^2 membrane. Taking the smaller of the two IP_3-activated unitary conductances (2 pA - Fadool and Ache, 1992b), a macroscopic current of average magnitude (39 pA; Figure 3-7) would require activation of as few as 20 channels, or only 1-6% of the available channels. Such a small fraction of recruited channels is consistent with the presumed sensory function of the cells (e.g. Firestein et al., 1991).

That the ability of the cultured cells to respond to odors did not correlate with the number or even the presence of processes, argues that the receptor sites for odors were not necessarily confined to the processes. At least in vertebrate ORNs, odor receptors are thought to be selectively localized to the cilia. Deciliating the frog olfactory epithelium with Triton X-100, for example, completely abolishes the electro-olfactogram (Adamek et al., 1984). Lowe and Gold (1991) confirmed this hypothesis by focally stimulating the cilia of dissociated salamander ORNs. Whether somatic sensitivity to odors in cultured lobster ORNs is induced or is typical of mature lobster ORNs *in situ* is unclear. Previous attempts to record responses to odors in freshly isolated somata have been consistently unsuccessful (e.g., Anderson and Ache, 1985), suggesting that somatic sensitivity to odors in cultured ORNs may have been induced by placing the cells in culture. This conclusion would be consistent with the observation that molluscan neurons raised in suspension culture to prevent process formation insert postsynaptic receptors otherwise normally confined to processes in the soma (Wong et al., 1981). On the other hand, Hatt (1990) finds odor-gated channels on the soma of freshly dissociated crayfish chemosensory neurons; channels that have the same structure-activity profiles and sensitivity of the intact cells. Ligand-gated histamine receptors on the soma of lobster ORNs resist the same enzymatic protocol used in the present study (McClintock and Ache, 1989a). Therefore, I cannot eliminate the possibility that the receptors in question normally occur on the somata of mature lobster ORNs, but are destroyed by the enzymatic treatment required to dissociate the cells and fail to recover in the subsequent 4-6 hr over which *in situ* recordings are usually made. It is known that enzymatic digestion used to prepare cells for patch clamping alters neurotransmitter responsiveness in invertebrate neurons (Oyama et al., 1990) and eliminates odor responsiveness in amphibian ORNs (Firestein and Werlin, 1989).

The latency to response was extremely brief, as short as 20 msec with the majority of the responses occurring within 125 msec of valve (spritzer) activation. These values fall below the lower limit of the range of latencies reported for dissociated salamander ORNs, the other system for which comparable measurements are available (175-600 msec--Firestein et al., 1990). Both systems lack a mucus barrier that would otherwise impede stimulus access to the receptor sites, so the latencies presumably reflect the actual time course of transduction. Breer et al. (1990) recently reported that levels of two second messengers in rat and insect ORNs, cyclic adenosine monophosphate and inositol 1,4,5-trisphosphate, respectively, peak in response to odor stimulation within 25-50 msec. Thus, while the latency of the response of cultured lobster ORNs is considerably shorter than that of salamander ORNs, it is consistent with second messenger-mediated transduction.

Thus, in this chapter it was shown that lobster ORNs not only survive in culture, but express the odor sensitivity and selectivity of their counterparts *in situ*, including the ability of odors to excite as well as inhibit the cells. As the cultured cells are morphologically and, presumably, electrotonically (as evidenced by the absence of a measurable equalizing time constant) more compact than their counterparts *in situ*, often consisting of only a spherical soma, the ability to characterize the transduction pathways and analyze potential interactions between them should be greatly facilitated *in vitro*.

CHAPTER 4
GTP-BINDING PROTEINS MEDIATE ODOR-EVOKED CURRENTS

Introduction

Heterotrimeric G proteins are a highly homologous set of proteins that relay an extracellular stimulus (neurotransmitter, hormone, photon, odorant) from cell-surface receptors to an effector (enzyme, ion channel) (Gilman, 1987; Neer and Clampham, 1988; Birnbaumer, 1992; Hille, 1992a, 1992b; Conklin and Bourne, 1993). To date there are genes encoding 16 α subunits, 4 ß subunits, and 5 gamma subunits, whose functions are incompletely known. Phylogenic trees assign G proteins into three families, α_s, α_q, and α_i, with similar functional correlates ascribed to subunits within a family group (Birnbaumer, 1992). The coupling of receptor type to effector is not known in many systems. Many of the criteria for the involvement of G proteins (Gilman, 1987) in olfactory transduction (Bruch, 1990; Shepherd, 1991; Reed, 1992; Ronnett and Snyder, 1992) have been satisfied: the requirement for GTP in the initiation of an odorant response (Pace et al., 1985; Pace

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NOTE: This chapter will be submitted to Chemical Senses and is formatted to meet its style regulations.

and Lancet, 1986), provoked responses by nucleotide analogues (Pace et al., 1985; Boekhoff and Breer, 1990; Breer et al., 1990), ribosylation by bacterial toxins (Pace et al., 1985; Bruch and Kalinoski, 1987; Boekhoff et al., 1990a, 1990b; Breer, 1991), immunochemical localization of individual G protein subtypes in the olfactory epithelium (Anholt et al., 1987; Jones et al., 1988; Mania-Farnell and Farbman, 1990), and finally molecular cloning of a highly specific olfactory G protein (Jones and Reed, 1989) as well as the discovery of putative olfactory receptors (Buck and Axel, 1991; Selbie et al., 1992; Ngai et al., 1993; Raming et al., 1993) that belong to a large superfamily of seven transmembrane receptors coupled to GTP-binding proteins.

The presence of G proteins across a broad range of species including toad, frog, rat, lobster, catfish, and insects (Pace and Lancet, 1986; Anholt et al., 1987; Bruch and Kalinoski, 1987; Jones and Reed, 1987, 1989; Jones et al., 1988; Boekhoff et al., 1990a; McClintock et al., 1990; Olson et al., 1992) gives credence to a transductory role in olfactory receptor neurons. Immunochemical, biochemical, and molecular evidence for G protein-linked mediation of olfactory signalling has in few instances been correlated with function (Jones et al., 1988; Jones, 1989; Mania-Farnell and Farbman, 1990; Raming et al., 1993). Representatives of all three G protein families have been localized in the olfactory epithelium, namely G_{olf}, G_s, G_o,

G_i, and $G_{\alpha 11}$ (Anholt et al., 1987; Jones and Reed, 1987, 1989; Jones et al., 1988, Mania-Farnell and Farbman, 1990; Shinohara et al., 1993; Abogadie and Bruch, in press; Rhoads et al., in press), but coupling of G protein subtypes to specific effector types remains incomplete. A direct method would be to study the involvement and type of G proteins in olfactory signalling by an electrophysiological approach, where changes in transduction current can be monitored. Lobster olfactory receptor neurons (ORNs) could provide an advantageous model, where multiple mechanisms of signal transduction are inferred by the dual polarity of odor-evoked current responses in single cells (McClintock and Ache, 1989b; Michel et al., 1992a; Fadool et al., 1993) and the presence of potential target effectors, inositol 1,4,5-trisphosphate (IP_3)- and cyclic monophosphate (cAMP)-gated ion channels, have been well studied (Fadool and Ache, 1992a; Michel and Ache, 1992).

Although the production of cyclic nucleotide and inositol phosphate second messengers can occur other than via G protein-mediated activation of adenylyl cyclase and phospholipase C (Wahl et al., 1989; Catt et al., 1991; Hille et al., 1992a; Majerus, 1992), preliminary evidence in our and other laboratories support the presence of G proteins in various lobster tissues (Miwa et al., 1990; Taggart and Landau, 1992) and in the olfactory organ of the lobster (Fadool et al., 1991a; McClintock et al., 1992). In this chapter, I now provide electrophysiological evidence for the involvement of a bacterial toxin insensitive G-protein in odor-evoked inward (excitatory) and outward (inhibitory) currents of whole cell voltage-clamped lobster ORNs. Secondly, through combined Western analysis and antibody perfusion of voltage-clamped whole cells, I have identified two candidate G protein subtypes of the α_i and α_q families linked functionally to the IP_3-mediated excitatory currents in these cells. As G proteins are a ubiquitous, evolutionarily conserved family of proteins among the multicellular eukarayotes (Pupillo et al., 1989; Simon et al., 1991), I feel my findings in this chapter can reveal insight into the potential ways in which G protein-linked, cell-surface receptors can be coupled to second messenger-gated ion channels in olfaction, as well as insight into the identity of potential G proteins mediating inositol phospholipid turnover in olfactory signalling of higher animals.

Methods

Animals

Adult specimens of the Caribbean spiny lobster, *Panulirus argus*, were collected in the Florida Keys and maintained in the laboratory in a flow-through seawater system. Animals were fed a mixed diet of frozen fish, squid, and shrimp.

Tissue Culture

The distinct clusters of olfactory (aesthetasc) receptor cells were dissected from the olfactory organ (lateral antennular filament) of adult specimens of the Caribbean spiny lobster, *Panulirus argus*. The clusters were enzymatically dissociated, and the resulting cells sustained in primary culture as described previously (Chapter 2). Briefly, the isolated clusters were incubated for 50 min at 80 rpm on an orbital shaker in 0.2 micron filter-sterilized solution of 2.5 mg papain and 12 mg L-cysteine in 10 ml *Panulirus* saline (PS) containing 1% penicillin, streptomycin sulfate, and amphotericin B (Gibco). Proteolytic digestion was stopped by replacing the enzyme solution with low glucose L-15 media supplemented with L-glutamine, dextrose, fetal calf serum, and BME vitamins. Cells were immediately plated on poly-d-lysine-coated glass coverslips. Cells were maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) at 24°C.

Electrophysiology

Patch electrodes were fabricated from 1.8 mm O.D. borosilicate glass and fire polished to a tip diameter of approximately 1.0 μm (bubble number 4.8; Mittman et al., 1987). High resistance seals of between 8 and 14 GΩ were obtained. Cells were viewed at 40X magnification with Hoffman optics. Signals were filtered at 5 kHz with a low-pass bessel filter and were analyzed by pCLAMP software (Axon Instruments).

Odor-activated currents were recorded in the whole-cell configuration with an integrating patch-clamp amplifier (Dagan 3900). In all experiments, cells were voltage-clamped at a holding membrane potential of -60 mV. Odors were "spritzed" on the cells from a seven barrel glass micropipette (Frederick haer) coupled to a single pressurized valve system (Picospritzer, General Valve) via a 6-way rotary valve as previously described (Chapter 2). Nonhydrolyzable forms of a G-Protein activator (GTP) and inhibitor (GDP), guanosine 5'-O-(3-thiotriphosphate) (GTPGammaS) and guanosine 5'-O-(2-thiodiphosphate) (GDPBetaS) respectively, were introduced to the cells through the patch pipette. To observe changes in odor-activated current in response to the above analogs, individual cells were sequentially patched. This allowed calculation of the initial odor responsivity, prior to the addition of the analog in the second patch on the same cell. In experiments using bacterial toxins, 1 μg/ml pertussis (PTX) or 50 μg/ml cholera toxin (CT) was added to the media of 36 h cultured cells for 30 h; a protocol similar to that used with other cultured systems (LaBelle and Murray, 1990; Okajima and Ui, 1984; Lambert and Nahorski, 1990). The magnitude of odor-activated currents of treated verses non-treated cells was compared. In additional studies, to insure PTX access into these cells, the active subunit of the PTX holotoxin, the A Protomer, was introduced (10 μg/ml; PTX A) to cells directly through the patch pipette. Change in odor-activated current at time 0 (control) was compared with that of time 5-10 min (treated) after break through to the whole-cell configuration.

In experiments where antibodies directed against various G-protein subunits were perfused into whole cells, patch electrodes were first tip-filled approximately 1 mm with patch electrode solution (see solutions, PE) and then backfilled with test antibody solution. Antibody solutions were prepared by an initial 1:25 dilution in PE, followed by brief vortexing, and then centrifugation for 30 min (Eppendorf, #12 setting). This reduced the likelihood of particulates blocking perfusion through the patch electrode. The antibody supernatant was subsequently diluted in PE to a 1:100 working concentration. In previous studies utilizing the same size pipette diameter, cell and antibody preparation, and recording conditions, evidence of perfusion of antibody generally occurred not later than 3 to 5 minutes (Chapter 5). Odor-evoked currents were monitored every min for up to 30 min so as to minimize adaptation or desensitization. Pipettes backfilled with PE or with non-immune IgG rabbit serum were used to monitor odor-evoked current over time in the absence of antibody perfusion.

For statistical comparisons, significance was defined at $p \leq 0.05$ for paired t-tests and at $\alpha = 0.05$ for analyses of variance.

Biochemistry

Mouse cerebellum (3 animals), lobster brain (3 animals), lobster olfactory receptor neuron (ORN) somata (8 antennules), and ORN dendrites (24 antennules) were isolated in chilled phosphate sucrose buffer (PSB). It was not possible to obtain enough isolated protein from cultured ORNs due to a high loss of tissue during removal of cells from substrate and the large number of live animals (≈ 100) required to test all G-protein probes. All dissected tissues were homogenized in chilled homogenization buffer (HB) 50 strokes by mortar and pestle (Wheaton, Size B) or 2 min by a Kontes tissue grinder in microcentrifuge tubes, and then tip sonicated (Heat systems, Ultrasonics Inc., W-220) three times for 15 s at a #3 setting while iced. Homogenates were centrifuged twice at 5,000 RPM (Eppendorf, #12 setting), 4°C for 30 min. Combined supernatants were then centrifuged (Beckman L70 Ultracentrifuge) 38,000 RPM for 2.5 h at 4°C. The recovered pellets were resuspended in HB by bath sonification (Heat systems, Ultrasonics Inc., W-225) three times for 15s at a #5 setting and frozen at -80°C until use.

Protein was determined by a Bradford photometric assay. 20-25 μg of the respective membrane preparations were separated at constant current (25 mA; 3 h) on a 10% homogenous SDS electrophoretic gel (0.55 mm thick, 29:1 acrylamide/bis-acrylamide). Proteins were transferred to nitrocellulose in normal blotting buffer (BB) at 0.5 mA for 4 h. Nitrocellulose membrane was soaked 2 x 10 min in Tween-tris buffered saline (TTBS). Non-specific binding to the membrane was blocked for 30 min with 2.5% Carnation dry milk in TTBS (Blotto). Nitrocellulose membrane was then incubated for 2.5h at RT with selected G-protein antibodies, washed 2 x 10 min in TTBS to remove unbound antibody, and reincubated for 2 h with a 1:250 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim). Electrophoretic bands were visualized by 4-chloro-1-naphthol color reagent activated by 30% H_2O_2 in tris buffered saline (TBS) and 20% methanol.

Solutions

PS consisted of (in mM): 458 NaCl, 13.4 KCL, 9.8 $MgCl_2$, 13.6 $CaCl_2$, 13.6 Na_2SO_4, 3 HEPES, and 2 glucose; pH 7.4. Modified L15 Media consisted of: 50 ml Liebowitz L15, 50 ml of 1.6X normal concentration of PS, 0.6g dextrose, 0.026 g L-glutamine, and 0.01% gentamicin. PSB consisted of (in mM): 10 phosphate and 250 sucrose; pH 7.3. HB consisted of (in mM): 320 sucrose, 10 Trizma base, 50 KCl, and 1 EDTA. TBS consisted of (in mM): 50 Tris, 150 NaCl; pH 7.5. TTBS was made by adding 0.1% Tween-20 to TBS. BB consisted of (in mM): 25 Tris, 0.1% SDS, 192 glycine, and 20% methanol. The patch electrode (PE) solution consisted of (in mM): 30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 180 K-acetate, and 696 glucose; pH 7.0. GTPGammaS (10^{-5}M) and GDPBetaS (10^{-5}M) were made daily in PE for electrical recordings. Odorants consisted of either single components (10^{-3}M) made daily in PS: taurine, betaine, l-proline, l-arginine, l-glycine, AMP, d-alanine or of a broad spectrum odorant mixture, TetraMarin (TET; commercial flake fish food, Tetra Werke, Melle, France), diluted 1000-fold in PS. A stock extract of TET was made by mixing 2 gm dry flakes in 60 ml saline, centrifuging the resulting suspension at 1400 g, filtering it through Whatman #3 filter paper, adjusting it to pH 7.4, and storing it frozen in 5 ml aliquots. Antibodies directed against the various G-protein subunits were diluted in Blotto at 1:250 or 1:1000 for Western analysis and were diluted in PE at 1:100 for electro-physiology. Anti-$G_{iß}$ and anti-$G_{o\alpha}$ were purchased from Upstate Biochemical and Dupont respectively. Immunopurified anti-$G_{olf\alpha}$ was a generous gift from Dr. Richard Bruch. Anti-G_q (Z811-5DE), made to the common carboxyl termini of α_q and α_{11} (the amino terminal cysteine and the last 12 amino acids, CILQLNLKEYNLV - Gutowski et al., 1991), was a generous gift from Dr. Paul Sternweis. Anti-G_q (E973), made to the amino acids 115-133 of the α_q peptide sequence and anti-$G_{\alpha 11}$ (E976), made to the amino acids 160-172 of the α_{11} sequence (Strathmann and Simon,

1990), were generous gifts from Dr. John Exton. The G-protein analogs, odorants, and all salts were obtained from Sigma Chemical. PTX A, PTX, and CT were obtained from Calbiochem.

Results

To test the involvement of GTP-binding proteins in odor transduction, a total of 19 cells were sequentially patched, incorporating either GTPGammaS or GDPBetaS in the second seal of the same cell. The magnitude of the odor-evoked inward (n=5) and outward current (n=5) was significantly increased when 10^{-5}M GTPGammaS was included in the pipette during odor stimulation (paired t-test). In contrast, the magnitude of each (n=5 inward, n=4 outward) was significantly decreased when 10^{-5}M GDPBetaS was included (paired t-test)(Figure 4-1). When the cultured cells were preincubated with 1 μg/ml PTX or 50 μg/ml CT for 30 h or when 10 μg/ml PTX A was introduced by way of the patch pipette, the magnitude of odor-evoked inward or outward current remained unchanged (one-way ANOVA, $\alpha \leq 0.05$, n= 5-10) among treated and untreated cells (Figure 4-2).

The antibodies directed against $G_{iß}$ and $G_{olf\alpha}$ demonstrated no positive immunolabelling at the expected reported molecular weights (arrows) in any of the lobster membrane preparations (Figure 4-3). Positive immunolabelling was observed in mouse cerebellum for anti-$G_{iß}$ at 35 kDa. This antibody exhibited a high degree of

Figure 4-1. Histogram of the mean odor-evoked inward and outward currents of voltage-clamped lobster olfactory receptor neurons (ORNs) that were sequentially patched. The magnitude of the odor-evoked current recorded with normal patch solution was normalized (solid bar) and compared to that recorded by the second patch on the same cell when GTPGammaS (striped bar) or GDPBetaS (open bar) was included in the patch pipette. Holding potential -60 mV. Number of sequentially patched cells as noted. * = significant difference, $p < 0.05$, paired t-test.

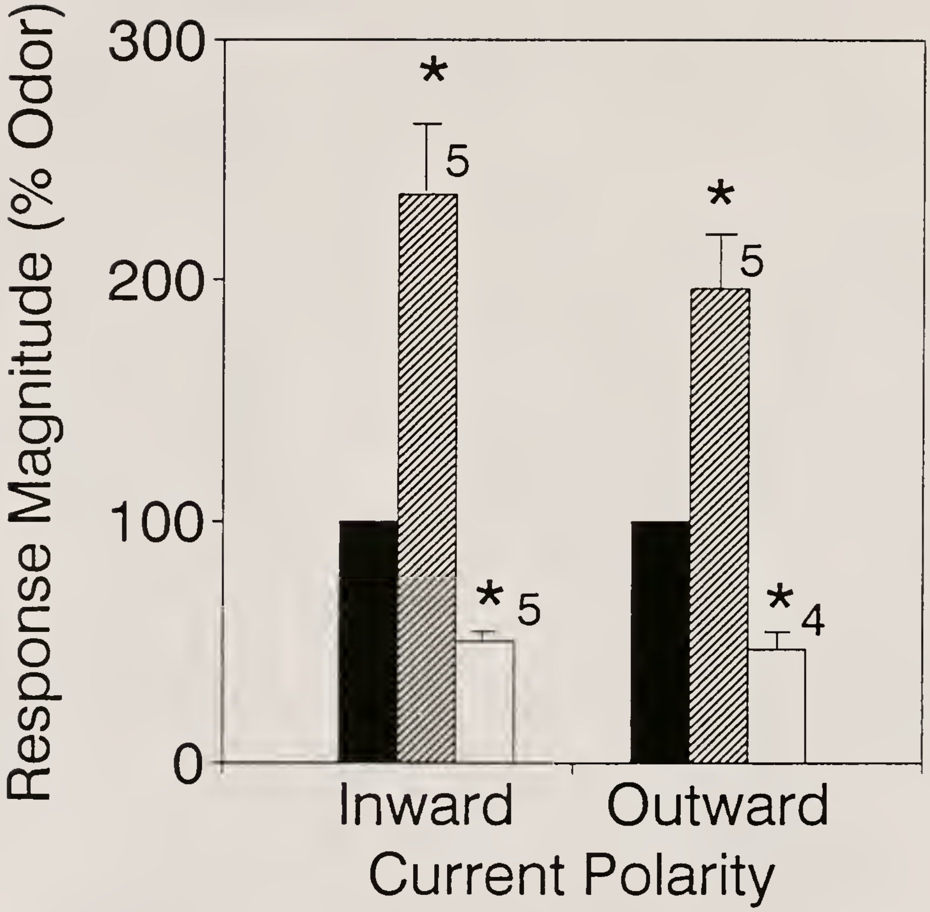

Response Magnitude (% Odor)
300
200
100
0
*
5
*
5
*
5
*
4
Inward
Outward
Current Polarity

Figure 4-2. Histogram of the mean inward and outward odor-evoked current of voltage-clamped lobster ORNs displayed as a percentage function of the control group normalized to 100. Solid bar = untreated cells, striped bar = 1 μM pertussis toxin (PTX) treated cells, open bar = 10 μM PTX A Protomer (PTX A) treated cells, checkered bar = 50 μM cholera toxin (CT) treated cells. No significant difference in treatment means (ANOVA, $\alpha \leq 0.05$, n= 5-10 as noted).

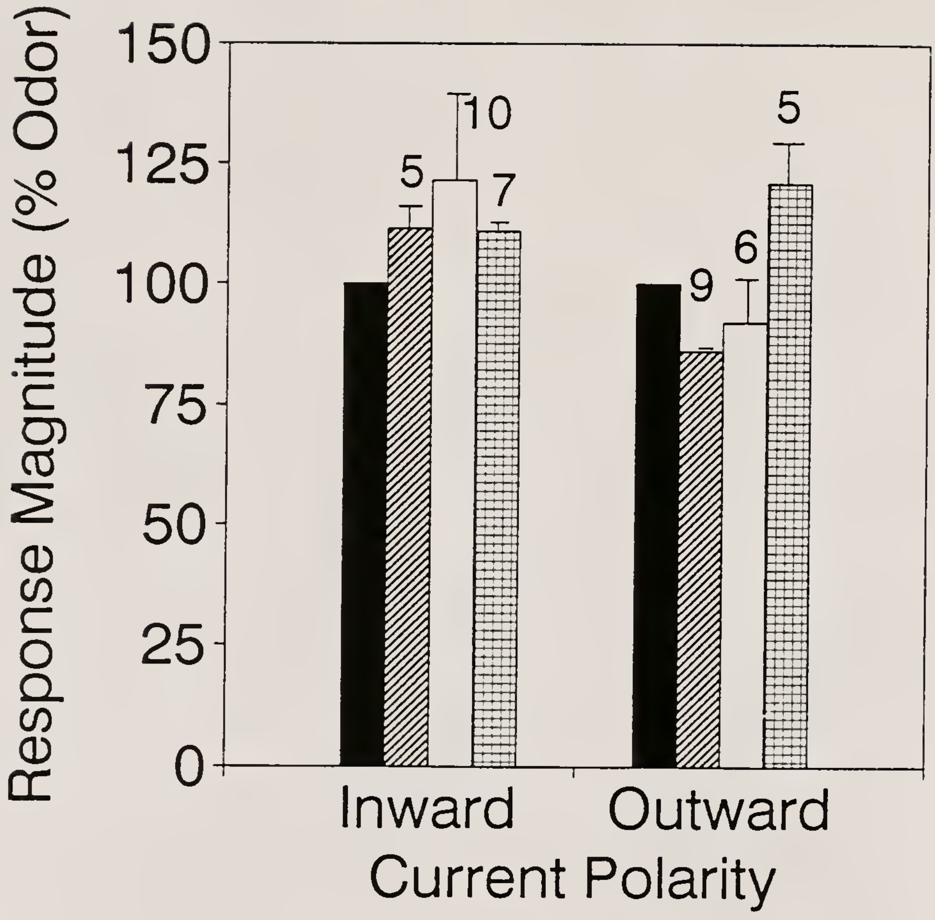
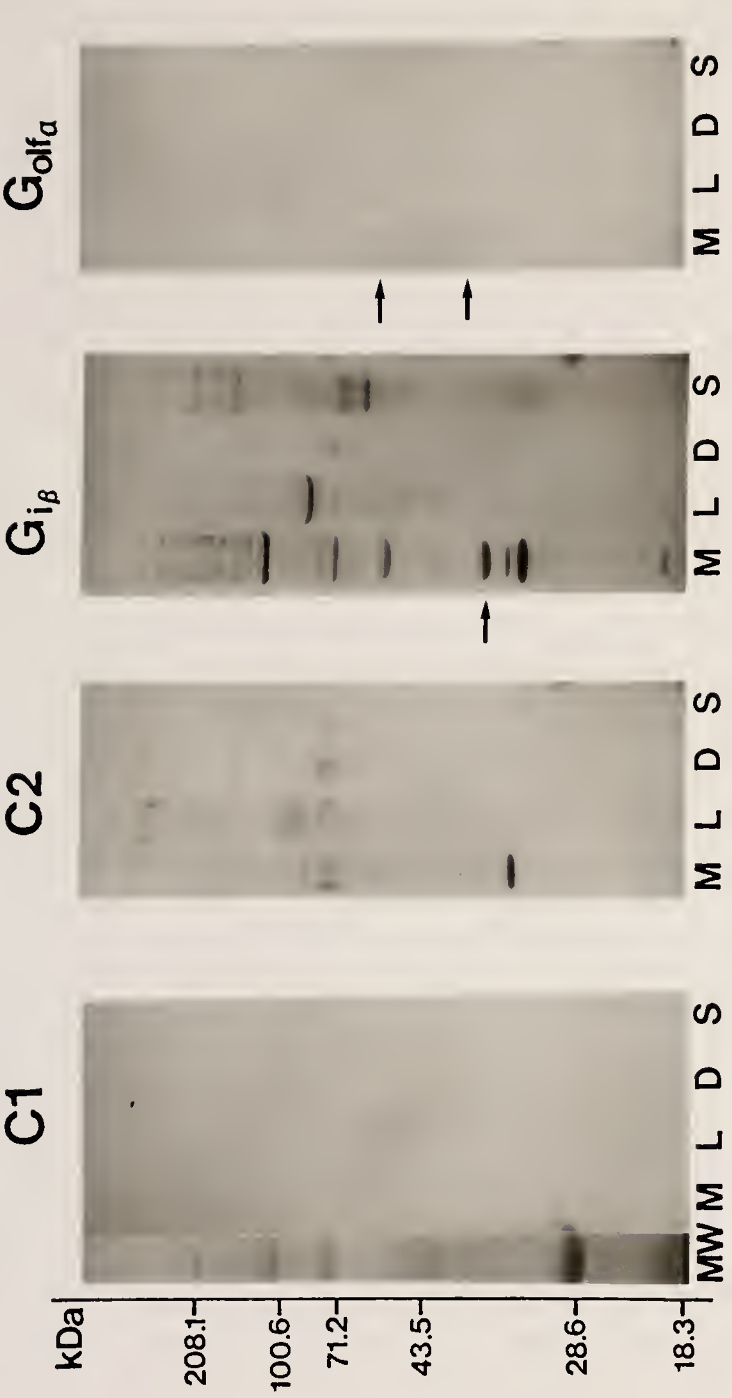

Response Magnitude (% Odor)
150
125
100
75
50
25
0
5
10
7
9
6
5
Inward
Outward
Current Polarity

Figure 4-3. Immunolabelling of membrane fractions from mouse cerebellum (M), lobster brain (L), lobster dendrites (D), and lobster receptor cell soma (S) to antibodies directed against G proteins classically coupled to adenylyl cyclase activation/inactivation. Molecular weight standards (MW) as indicated. Arrow denotes the expected reported molecular weight (see Figure 4-5) of the G protein subtype being tested. Control 1 (C1) = omission of primary antibody; Control 2 (C2) = substitution of non-immune rabbit IgG for primary antibody. $G_{i\beta}$ 1:250, $G_{olf\alpha}$ 1:1000.


C1
C2
G_{i_β}
G_{olf_α}
MW
M
L
D
S
kDa
208.1
100.6
71.2
43.5
28.6
18.3

non-selective staining at higher molecular weights (60-210 kDa) than those reported for any G-proteins. This was true for all tissues, including that isolated from either mouse or lobster. The antibody directed against $G_{o\alpha}$ demonstrated immunolabelling of a 40.5 kDa peptide in mouse cerebellum, lobster brain, and lobster dendrite (Figure 4-4), close to the expected, reported molecular weight (arrow) of 39 kDa for this protein subunit. No immunolabelling at this molecular weight was observed in extracts from lobster somata. Again a degree of non-selective staining of high molecular weight proteins was observed for this antibody. Anti-G_q (E973) and anti-$G_{\alpha 11}$ (E976) displayed no positive immunolabelling in any of the lobster tissues at the expected, reported molecular weights for these protein subunits (arrows), and only slight immunolabelling in mouse cerebellum by anti-G_q (E973) at 42 kDa (Figure 4-4). Anti G_q (Z811-5DE) was not tested by Western analysis. In no case were protein bands observed when the primary antibody was omitted (Figure 4-3; C1). Background staining of high molecular weight proteins in both mouse and lobster was observed when the primary antibody was replaced with non-immune rabbit IgG (Figure 4-3; C2). A summary of all Western analyses can be found in Figure 4-5.

Because the odor-evoked macroscopic inward and outward currents in lobster ORNs are mediated by the second messengers, IP_3 and cAMP respectively (Fadool and Ache,

Figure 4-4. Immunolabelling of membrane fractions from mouse cerebellum (M), lobster brain (L), lobster dendrites (D), and lobster receptor cell soma (S) to antibodies directed against G proteins classically coupled to phospholipase C activation. Molecular weight standards (MW) as indicated. Arrow denotes the expected reported molecular weight (see Figure 4-5) of the G protein subtype being tested. $G_{o\alpha}$, $G_{q\alpha}$(E973), and $G_{11\alpha}$ = 1:250.

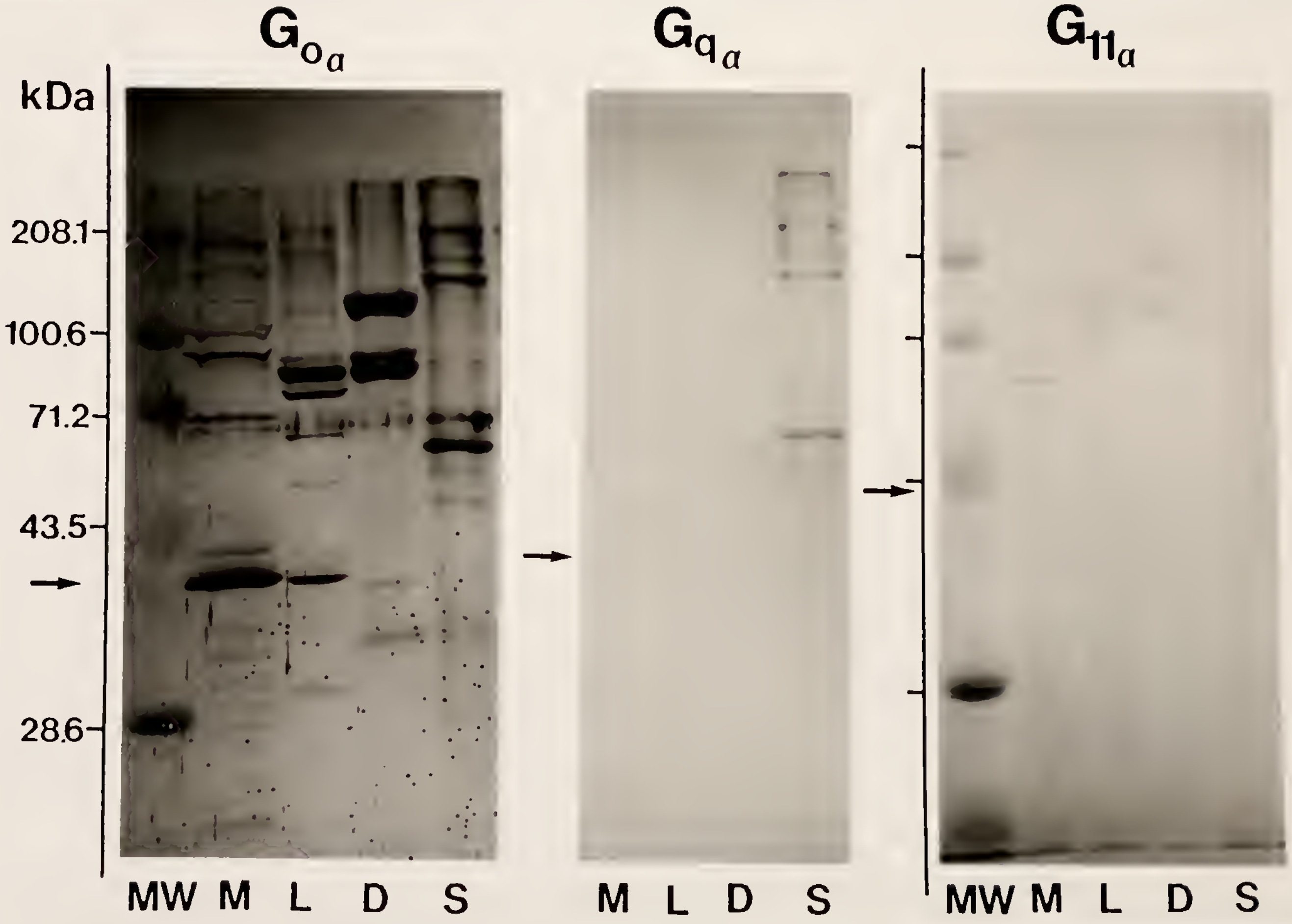
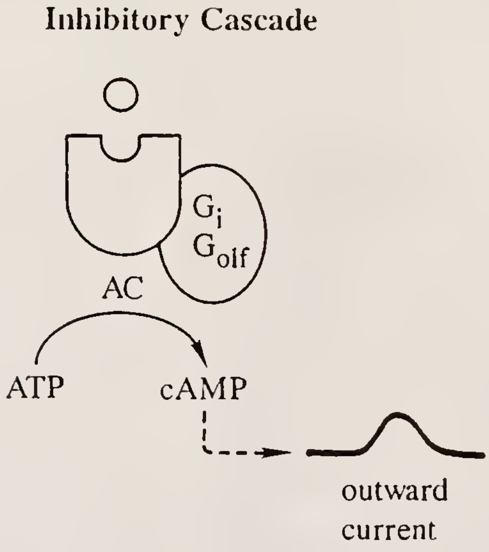
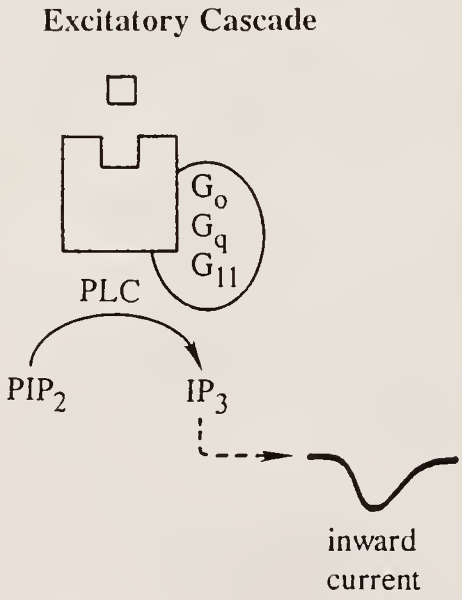

G_{o_α}
G_{q_α}
G_{11_α}
kDa
208.1
100.6
71.2
43.5
28.6
MW
M
L
D
S
M
L
D
S
MW
M
L
D
S

Figure 4-5. G-protein subtypes localized in mouse and lobster tissues and the theoretical G-protein linked effectors in lobster olfactory receptor neurons (ORNs). TOP: Summary table of the cross-reactivity of antibodies directed against various G-protein subtypes as shown by the Western analyses of Figures 4-3 and 4-4. "+" = positive immunoreactive protein;"-" = no apparent immunoreactivity. BOTTOM: Diagrammatic representation of all hypothesized transduction cascades in lobster ORN based upon known receptor-effector coupling reported in other systems. Inhibitory cascade: Odor molecule (circle) binds to a cell-surface receptor linked to either G_i or G_{olf} protein to activate adenlyly cyclase (AC), which converts adenosine trisphosphate (ATP) to cyclic monophosphate (cAMP), which directly gates (------>) an ion channel evoking an outward (inhibitory) transduction current. Excitatory cascade: Odor molecule (square) binds to a cell-surface receptor linked to either G_o, G_q, or G_{11} protein to activate phospholipase C (PLC), which converts phosphoinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3), which directly gates (------>) an ion channel evoking an inward (excitatory) transduction current.

Protein	Reported MW (kDa)	Tissue			
		Mouse Brain	Lobster Brain	ORN Dendrite	ORN Somata
$G_{i\beta}$	35-36	+	-	-	long smear
G_{olf}	43 & 52	-	-	-	-
$G_{o\alpha}$	39	+	+	+	-
G_q	42	+	-	-	-
$G_{11\alpha}$	42	-	-	-	-





1992a; Michel and Ache, 1992), binding of antibodies to the G-proteins that target the enzymatic production of these second messengers might be expected to perturb the transduced current (Figure 4-5). In antibody perfusion experiments, the perfusion of anti-$G_{olf\alpha}$, anti-$G_{o\alpha}$, anti-$G_{i\beta}$, anti-G_q (Z811-5DE), anti-G_q (E973), or anti-$G_{\alpha 11}$ (E976) or the perfusion of non-immune rabbit IgG or normal PE had no effect on the magnitude of odor-evoked outward currents. In contrast, binding of either anti-G_q (Z811-5DE) or anti-$G_{o\alpha}$, completely blocked the odor-evoked inward current within several minutes. It was not possible to reverse the effect of the antibody block by inclusion of the peptide (in the patch pipette) to which the antisera were raised because this made the ORN membranes too unstable for reliable recording. Cells perfused with non-immune rabbit IgG or normal PE typically showed no decay in odor-evoked outward current over the tested time interval but displayed a rundown of odor-evoked inward currents over this same period. Even in these control conditions, however, a measurable inward current could be detected in many cells up to 1 hr. An example of the odor-evoked responses observed during perfusion of anti-G_o is shown in Figure 4-6.

The effect of antibody perfusion on the magnitude of odor-evoked currents was fairly consistent across a sample population (Figure 4-7). Binning the measured odor-evoked currents into 5 minute intervals and normalizing an

Figure 4-6. Whole cell voltage-clamp recordings of odor-evoked inward (inward) and outward (outward) currents as a function of time and perfusion treatment in lobster olfactory receptor neurons (ORNs). Shown are current recordings from 2 ORN, the first cell (left) was perfused with non-immune rabbit IgG (rabbit serum control) and the second cell (right) was perfused with an antibody directed against the α subunit of G_o protein (anti-$G_{o\alpha}$). Membrane potential = -60 mV; 1:100 dilution of serum or antibody; arrow denotes application of the odor.

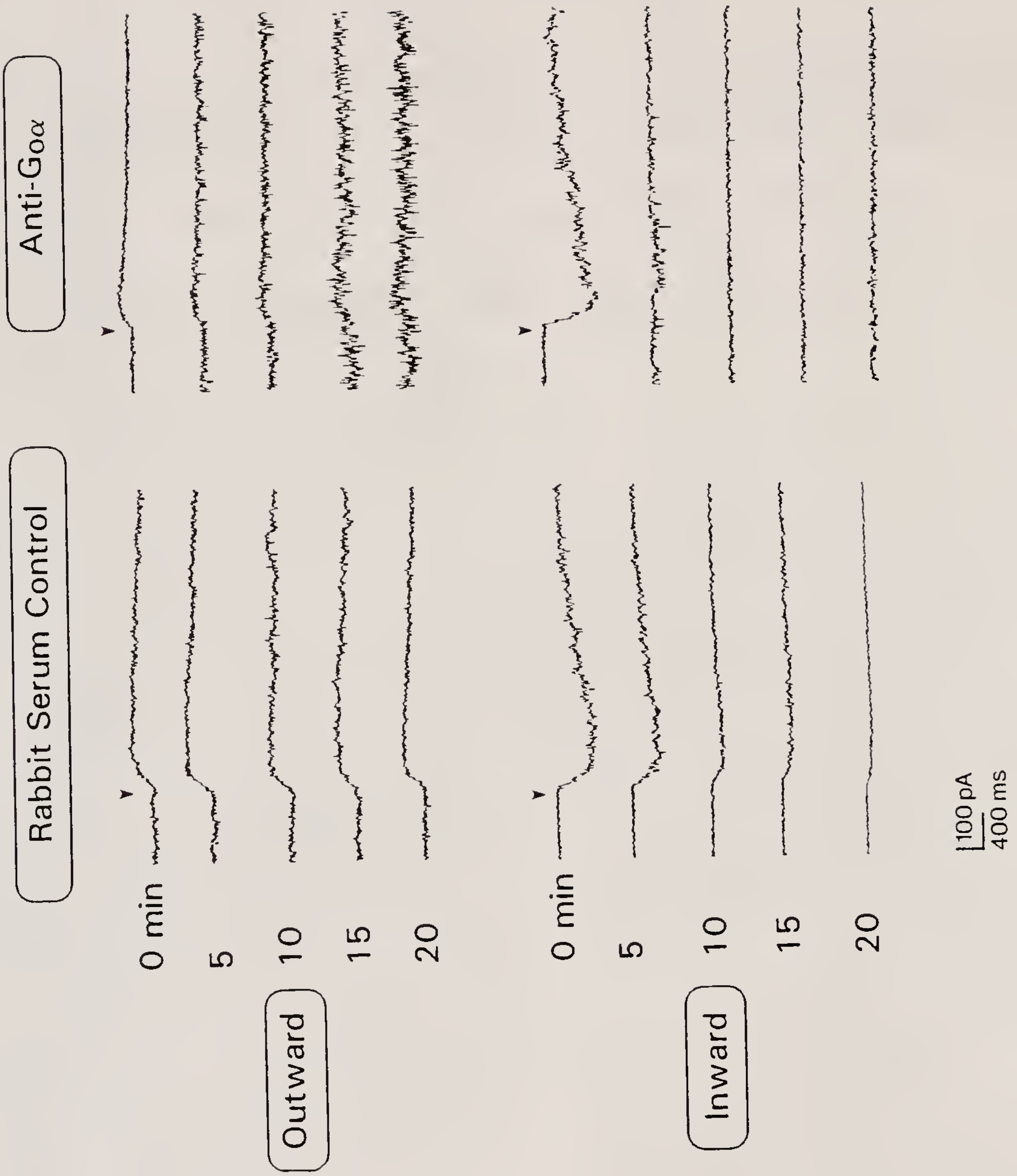
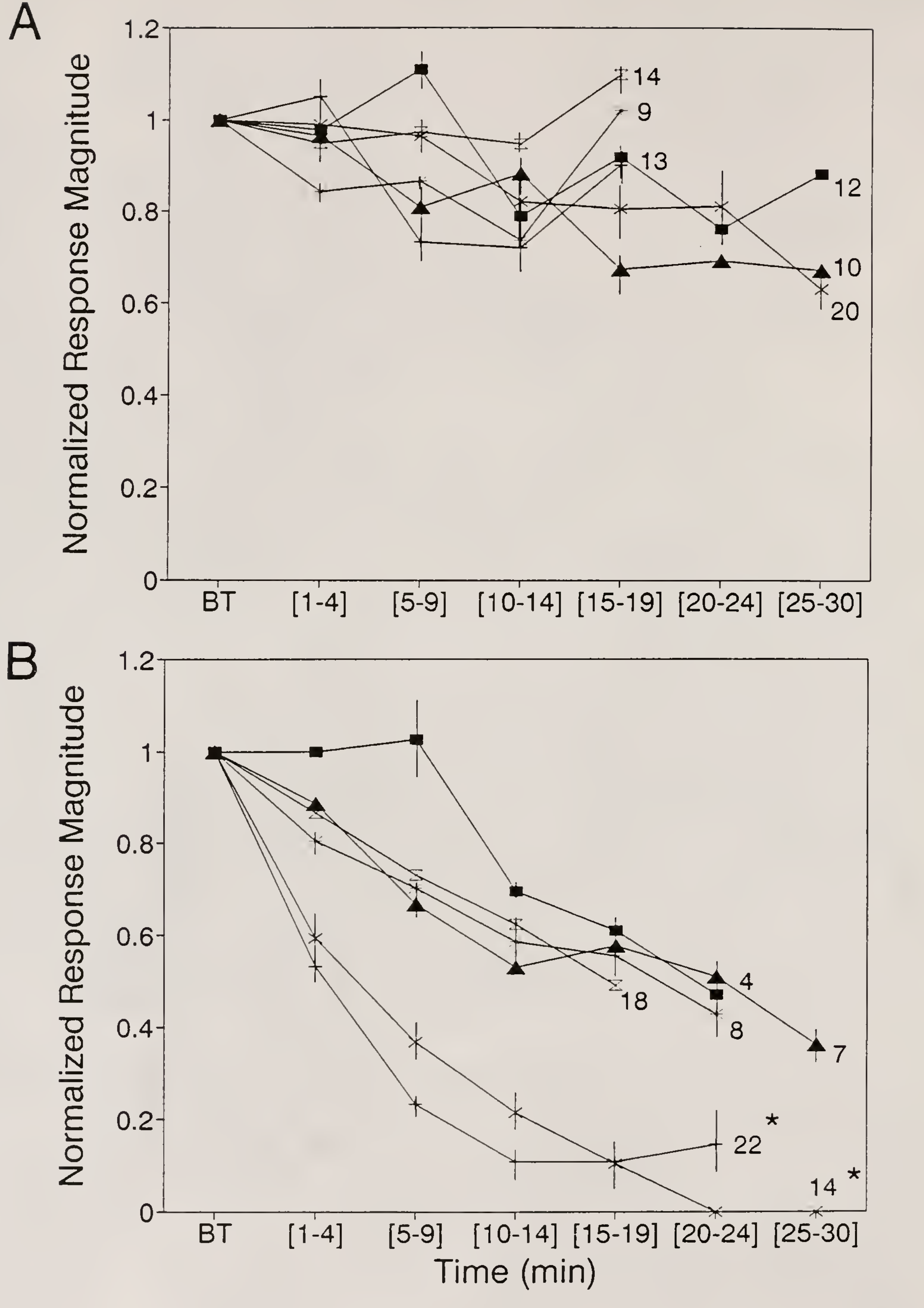

Rabbit Serum Control
Anti-Goα
Outward
Inward
0 min
5
10
15
20
0 min
5
10
15
20
100 pA
400 ms

Figure 4-7. Line graphs of the mean outward (A) and inward (B) odor-evoked currents as a function of time and perfusion treatment in whole-cell voltage-clamped lobster olfactory receptor neurons (ORNs). Each data point represents the mean (± SEM) odor-evoked current normalized to that at breakthrough (BT) to the whole-cell configuration and binned into the bracketed time intervals. Number of repetitions as noted. * = significant difference across treatment dimension (blocked factorial design two-way ANOVA, $\alpha \leq 0.05$). Membrane potential = -60 mV.
--*-- = serum, --▲-- = patch solution, --■-- = anti-G_{olf},
--+-- = anti-G_o, --X-- = anti-G_i, --x-- = anti-G_q


A
Normalized Response Magnitude
1.2
1
0.8
0.6
0.4
0.2
0
BT
[1-4]
[5-9]
[10-14]
[15-19]
[20-24]
[25-30]
14
9
13
12
10
20
B
Normalized Response Magnitude
1.2
1
0.8
0.6
0.4
0.2
0
4
18
8
7
22 *
14 *
BT
[1-4]
[5-9]
[10-14]
[15-19]
[20-24]
[25-30]
Time (min)

individual olfactory neuron response to its initial response magnitude upon break through to the whole-cell configuration, indicated that the binding of anti-$G_{olf\alpha}$, anti-$G_{o\alpha}$, anti-$G_{i\beta}$, anti-G_q (Z811-5DE), anti-G_q (E973), or anti-$G_{\alpha 11}$ (E976) or the perfusion of non-immune rabbit IgG or normal PE had no affect on the magnitude of odor-evoked outward current across either the time or treatment group dimension (blocked factorial design two-way ANOVA; Figure 4-7A). In a similar analysis of odor-evoked inward currents, all test perfusions, including controls, exhibited a decrease in response magnitude across the time dimension, but only those cells perfused with anti-$G_{o\alpha}$ or anti-G_q (Z811-5DE) showed a significant decrease in response magnitude compared across treatment groups (blocked factorial design two-way ANOVA; Figure 4-7B).

Discussion

The finding that nonhydrolyzable analogs of GTP and GDP respectively increase and decrease both the macroscopic odor-evoked inward (excitatory) and outward (inhibitory) currents in lobster ORNs strongly supports a transductory role for G proteins in both conductance pathways. Pertussis and cholera toxin incubation of cultured ORNs did not perturb the magnitude of the odor-evoked currents, implying that the G protein(s) involved in transduction were of the bacterial-toxin insensitive class. The ability of a G protein to undergo bacterial toxin ADP-ribosylation is an indication of its subtype: members of the α_i family, including $G_{\alpha i}$ and $G_{\alpha o}$, can be ribosylated by PTX (Katada and Ui, 1982; Codina et al., 1983; Bokoch et al., 1984; Neer et al., 1984; Sternweis and Robinshaw, 1984); members of the α_s, including $G_{\alpha s}$ and $G_{olf\alpha}$, can be ribosylated by CT (Gill and Meren, 1978); and members of the α_q family, including $G_{\alpha 11}$ and G_q, lack the recognition site for ribosylation (Pang and Sternweis, 1990; Strathmann and Simon, 1990; Blank et al., 1991; Sternweis et al., 1992). Since PTX is known to alter odor-evoked second messenger production in rat and insect olfactory ciliary preparations (Boekhoff et al., 1990a) and ribosylates a 37/39 kDa doublet and 3 lower molecular weight proteins in lobster aesthetasc membrane preparations (McClintock et al., 1990), I questioned whether the toxin was penetrating the ORN cell membrane, albeit at micromolar concentrations and for 30 hours of incubation. I eliminated this possibility by injecting the active subunit of the PTX holoenzyme, the A Protomer (PTX A), into whole-cells via the patch pipette, using similar tip diameters and perfusion times documented for voltage-clamp studies of B5 snail neurons and frog sympathetic neurons (Elmslie, 1992; Man-Son-Hing et al., 1992). Even though lobster aesthetasc membranes contain a PTX-ribosylated protein(s) (McClintock et al., 1990) and a putative $G_{\alpha i}$ subunit has been cloned from lobster containing a potential cysteine ribosylation recognition site (McClintock et al., 1992), my data support functionally, bacterial toxin-insensitive G-proteins most probably similar to those of the α_q family. In contrast to PTX, the ability for CT to ribosylate G proteins in lower metazoans has been questionable, as these animals do not possess an essential lipid component in their plasma membrane to make such a modification (John Schetz, unpub data). It is thus less clear whether the lack of CT sensitive odor-evoked currents in lobster ORNs should be truely classified as CT-insensitive.

Selective block of odor-evoked inward currents by the binding of antibodies directed against $G_{o\alpha}$ and $G_{q\alpha}$ is consistent with the $InsP_3$ mediation of excitation in these neurons (Chapter 5). $PLCß_1$ is the reported effector for both of these G protein subtypes (Review: Berstein et al., 1992; Sternweis and Smrcka, 1992; Reports: Moriarty et al., 1990; Blank et al., 1991; Smrcka et al., 1991; Taylor et al., 1991), inferring that the lobster possesses a similar isoform of this lipase to generate $InsP_3$ from PIP_2. It is interesting to note that mRNA encoding G_o is enriched in rat olfactory epithelium (Jones et al., 1990) and that G_o protein was suspected to be coupled to $InsP_3$-mediated odor detection based on bacterial-toxin sensitivity in this same organism (Breer, 1990).

If both a $G_{q\alpha}$- and a $G_{o\alpha}$-like G protein mediates $InsP_3$ turnover in lobster ORNs, why the apparent redundancy? Most recently are the report of two different G protein subtypes mediating inositol phospholipid turnover in other systems (Voyno-Yasenestskaya et al., 1989; Gerwins and Fredholm, 1992). Of particular interest are identified G protein subtypes in smooth muscle, where both a toxin sensitive and toxin insensitive protein are distinguished through separate receptor activation by adenosine and bradykinin (Gerwins and Fredholm, 1992). The coexpression of $G_{q\alpha}$ and $G_{o\alpha}$ subtypes within single lobster ORNs could explain the apparent bacterial-toxin insensitivity of the odor-evoked inward currents: even though $G_{o\alpha}$ is potentially ribosylated, rendering the G protein of the transduction cascade in a GDP-bound inactive state (Okajima and Ui, 1984), the active odorant transduction cascade utilizing $G_{q\alpha}$ is unaffected and no change in current magnitude is measurable.

The recognition of a 40.5 kDa protein by anti-$G_{o\alpha}$ specifically in the dendritic membrane preparation rather than in the isolated somata of lobster ORNs, is in keeping with the olfactory transduction elements being confined to the cilia or the analogous outer dendritic membrane in crustaceans (Nakamura and Gold, 1987; Kurahashi, 1989; Firestein et al., 1990). The binding of an antibody to its epitope does not confer perturbation of physiological function. Only anti-$G_{q\alpha}$ (Z811-5DE) and not anti-G_q (E973) had been previously demonstrated to target PLC activity, as witnessed by its ability to attenuate the hormone stimulated turnover of PIP_2 (Gutowski et al., 1991). As anti-$G_{o\alpha}$ was commercially acquired, the mechanism of its discovered block of excitatory currents is unknown. It cannot be excluded that antibody binding of G_o acts to perturb a membrane-delimited modulation of ion channels (Brown, 1991; Hille, 1992a), instead of perturbing the function of the enzyme effector (PLC). Interfering with a mechanism that alters the gating of K^+ channels, such as that reported by $G_{o\alpha1}$, would be expected to target the odor-evoked outward currents that are mediated by an underlying K^+ conductance (Michel et al., 1991) in these ORNs, however, these currents do not change with any anti-G_o perfusion. If the lobster $G_{o\alpha}$ subunit was more like that of a $G_{o\alpha2}$ subtype, then binding of anti-G_o could be suspected to interfere with a mechanism that alters the gating of Ca^{2+} channels, such as that of the 74 pS $InsP_3$-gated channel (Chapter 6), which would be consistent with the observed block in odor-evoked inward current.

Future experiments will certainly be directed at sorting the complexity of the transduction elements in lobster ORNs: neurons that contain more that one odor-evoked conductance, G-protein mediated response, second messenger, and even more than one type of ion channel gated by a given second messenger. Earlier electrophysiological findings (see Introduction), recent evidence for at least two and maybe three second messenger-gated ion channels in the outer dendrites of ORNs *in situ* (Ache et al., in press), and biochemical cross-receptor binding studies (Michel et al., 1992b) do not well support an emerging theory in the chemoreceptive field of one receptor per olfactory receptor cell (Lancet et al., 1993). Two different G-proteins mediate inward odor-evoked currents and presumably a third as yet unidentified G-protein must mediate the outward odor-evoked currents. In a monoclonal exclusion model, as proposed by Lancet et al. (1993), this would require a single cell-surface receptor type to be linked to two or three different G protein subtypes. Even greater levels of complexity are probable when considering ßΓ subunit exchange (Casey et al., 1988), potential functioning of ßΓ dimers (Birnbaumer, 1992; Taussig et al., 1993) and second messenger related cross-talk (Bouvier, 1990). The potential sorting of G protein-linked transduction cascades across cells or the interaction of cascades within a cell may provide an avenue for amplification and diversity in the output of an ORN.

CHAPTER 5
IP_3-ACTIVATED CHANNELS IN THE PLASMA MEMBRANE

Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) is now well established as a second messenger in olfactory transduction (reviews: Anholt, 1991; Firestein, 1991). Since odors rapidly and transiently elevate levels of inositol 1,4,5-trisphosphate (IP_3) in the cilia/outer dendritic membranes of olfactory receptor neurons (ORNs) in fish (Huque and Bruch, 1986), rats and insects (Breer et al., 1990), IP_3 must also be considered as an olfactory second messenger.

The relationship between phospholipid and cyclic nucleotide second messengers in olfactory transduction is still obscure. Odors that elevate IP_3 in ciliary membrane preparations of rat ORNs fail to elevate cAMP, and vice versa (Boekhoff et al., 1990b; Breer and Boekhoff, 1991), suggesting that the two second messengers mediate different, odor-specific transduction pathways. Indeed, two distinct transduction pathways can be predicted in lobster and amphibian ORNs, where odors have been shown to suppress as

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well as excite the cells via separate conductances (McClintock and Ache, 1989b; Michel et al., 1991; Dionne, 1992). In lobster ORNs, cAMP mediates an inhibitory transduction pathway that suppresses the output of the cell (Michel and Ache, 1992b). Given that IP_3 has been implicated as an olfactory second messenger in at least one other species of arthropod (Breer et al., 1990), IP_3 is a logical candidate to mediate excitation in the lobster, but the excitatory transduction pathway in lobster ORNs is unknown.

IP_3 is known to release Ca^{2+} from non-mitochondrial intracellular stores by binding to a receptor protein that contains both an IP_3 recognition site and a Ca^{2+} channel (review: Ferris and Snyder, 1992). It is unclear whether such IP_3 receptors are associated with the plasma membrane in neurons (Worley et al., 1987; Maeda et al., 1989, 1991; Mignery et al., 1989; Ross et al., 1989), although IP_3 receptors occur in the plasma membrane of lymphocytes (Kuno and Gardener, 1987; Khan et al., 1992), mast cells (Penner et al., 1988), and in transverse tubules (Viven and Coronado, 1988). Evidence is beginning to implicate what is perhaps a novel type of IP_3 receptor in the plasma membrane of ORNs. IP_3 activates a channel reconstituted from the cilia of catfish ORNs (Restrepo et al., 1990). The cilia are enriched in a 107 kDa protein that binds radiolabelled IP_3, but whose molecular weight and affinity for IP_3 are less than those reported for intracellular cerebellar IP_3 receptors (Kalinoski et al., 1992). Preliminary evidence localizes immunoreactivity of an antibody directed against cerebellar IP_3 receptors to the cilia of rat ORNs (Cunningham et al., 1992). As the cilia of ORNs are devoid of organelles, it could be assumed that the target of this second messenger in olfactory neurons is a plasma membrane IP_3 receptor.

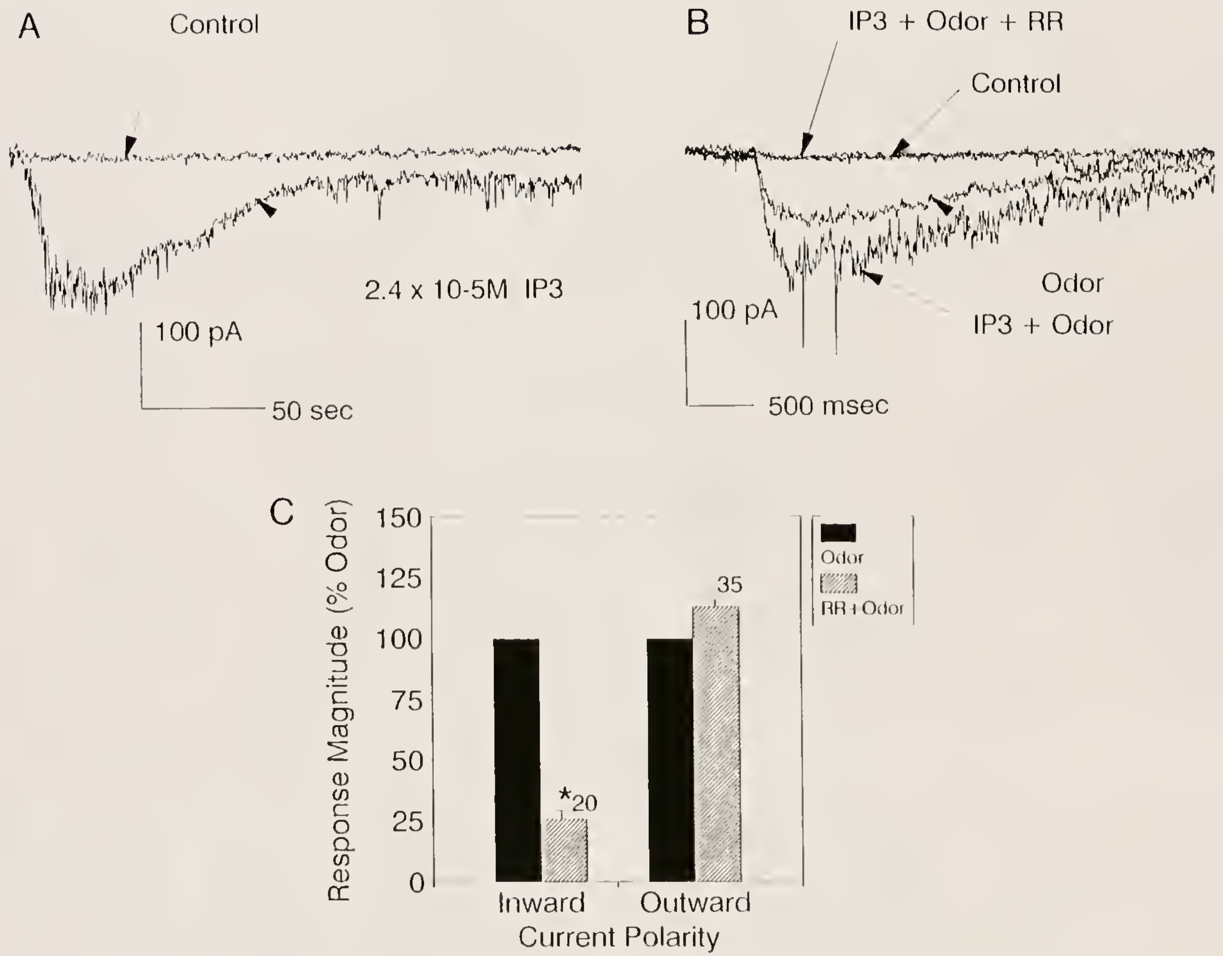
Here, in this chapter I report that IP_3 mediates excitation in cultured lobster ORNs by directly gating ion channels in the plasma membrane. The study provides functional evidence for channels activated by IP_3 in the plasma membrane of neurons.

Results

Macroscopic Currents

Introducing 2.4×10^{-5} M IP_3 into the cells through the patch pipette evoked a prolonged, inward current in 17 of 41 (42%) cells, with an average peak amplitude of 35.1 ± 10.4 pA (Figure 5-1A). Without IP_3 in the pipette, the cells held a steady baseline over the test interval of 4 min. These particular cells were not tested for their ability to respond to odors, but the most effective odor I have been able to test, an extract of fish food (TET - see Experimental Procedures), excites approximately 37% of cultured ORNs (see Chapter 3). The percentage of cells activated by introducing IP_3 through the pipette, therefore,

Figure 5-1. Inositol 1,4,5-trisphosphate (IP_3)- and Odor-Evoked Macroscopic Currents in Voltage-Clamped Cultured Lobster Olfactory Receptor Neurons (ORNs)
A) Whole-cell recording from an ORN that was sequentially patched with normal patch solution, and then IP_3, in the patch pipette. Holding potentials, -60 mV.
B) Whole-cell recording from an ORN that was sequentially patched with normal patch solution, and then IP_3, in the patch pipette and "spritzed" in each instance with odors. 1st patch: Control, response to recording media. Odor, response to TetraMarin (TET). 2nd patch: IP_3 + Odor, response to TET with 2.4×10^{-5} M IP_3 in the patch pipette. IP_3 + Odor + RR, response to TET with IP_3 in the patch pipette while bathing the cell in 10 μM ruthenium red (RR). Holding potentials, -60 mV.
C) Plot of the mean peak amplitude of odor-evoked inward (n=20) and outward (n=35) currents in the absence (solid bar) and presence (striped bar) of 10 μM RR in the bath. Response magnitudes in the absence of RR were normalized to 100%. * = significant difference at $p \leq 0.05$, paired t-test. Holding potential, -60 mV.


A
Control
2.4 x 10-5M IP3
100 pA
50 sec
B
IP3 + Odor + RR
Control
Odor
IP3 + Odor
100 pA
500 msec
C
Response Magnitude (% Odor)
150
125
100
75
50
25
0
*20
35
Inward
Outward
Current Polarity
Odor
RR+Odor

is consistent with the percentage of cells that would be expected to be excited by odors.

The polarity of the IP_3-induced current matched the polarity of the current induced by TET (Figure 5-1B). Introducing 2.4×10^{-5} M IP_3 through the patch pipette increased the magnitude of the TET-evoked inward current to 188 ± 12% of that evoked by TET without IP_3 in the pipette (n=4 sequentially patched cells) (Figure 5-1B). It was not determined if higher concentrations of IP_3 could saturate the odor-evoked inward current. The inward current induced by TET + IP_3 in all four instances was substantially blocked by bathing the cells with 10 μM ruthenium red (RR - a drug reported to block some IP_3-gated conductances: Ehrlich and Watras, 1988; Berridge, 1989), supporting a common origin of the IP_3- and odor-induced currents (Figure 5-1B).

The effect of RR was selective for the inward current (Figure 5-1C). Bathing the cells in 10 μM RR significantly reduced the peak amplitude of the inward current evoked by TET, proline, or betaine from an average of 19.1 ± 4.0 to 4.8 ± 4.3 pA (n=20, paired t-test). The drug, however, had no significant effect on the peak amplitude of the outward current evoked by proline, betaine, glycine or taurine, which averaged 16.4 ± 2.6 pA before, and 15.5 ± 6.9 pA after, bathing the cells in 10 μM RR (n=35, paired t-test).

Unitary Currents

TET transiently activated unitary currents in four of 21 cell-attached recordings (Figure 5-2). The mean estimated chord conductance for the four channels was 86.7 ± 17.1 pS (Table 5-1). The probability of being open (Pr_{open}) for the four odor-activated channels increased from an average of 0.02 ± 0.001 to 0.11 ± 0.03. The channels characteristically had "flickery" kinetics and opened in long bursts averaging 72.2 ± 53.6 msec. The mean open (t_o) and closed (t_c) times for the four odor-activated channels were best fit by double exponentials (Table 5-1).

Applying 10^{-7} M IP_3 to the inside face of 86 cell-free patches activated unitary currents in 63 of the patches within 100 msec of application. The patches typically contained 1-3 channels, although 4 and 5 channels could be resolved in two of the patches, respectively. The channels were of two different types; in only one instance were both types observed in a single patch of membrane. One type of channel (Figure 5-3A) had a mean slope conductance of 73.7 ± 5.7 pS and reversed polarity at 2.4 ± 2.2 mV in symmetrical solutions (n=12) (Figure 5-3B). The open probability function closely followed a Gaussian distribution (Figure 5-3C). The Pr_{open} was between 0.04 and 0.05 from -90 mV to +60 mV (Figure 5-3B, inset). The mean open (t_o) and closed (t_c) times were best fit by double exponentials (n=20)(Figure 5-3D, Table 5-1).

Figure 5-2. Odor-evoked Unitary Currents in a Cell-Attached Patch from a Cultured Lobster Olfactory Receptor Neuron (ORN)
Control: Unitary currents recorded in response to "spritzing" an ORN with normal patch solution to depolarize it.
+ Odor: Unitary currents recorded in response to "spritzing" an ORN with the odor TetraMarin (TET). In both upper and lower traces, membrane potential, -60 mV. Records filtered at 2 kHz. In this and subsequent figures, "C" denotes closed state of the channel whereas "O" denotes open state of the channel. Superposition in the open state of multiple channels in a patch are numbered in subscript.

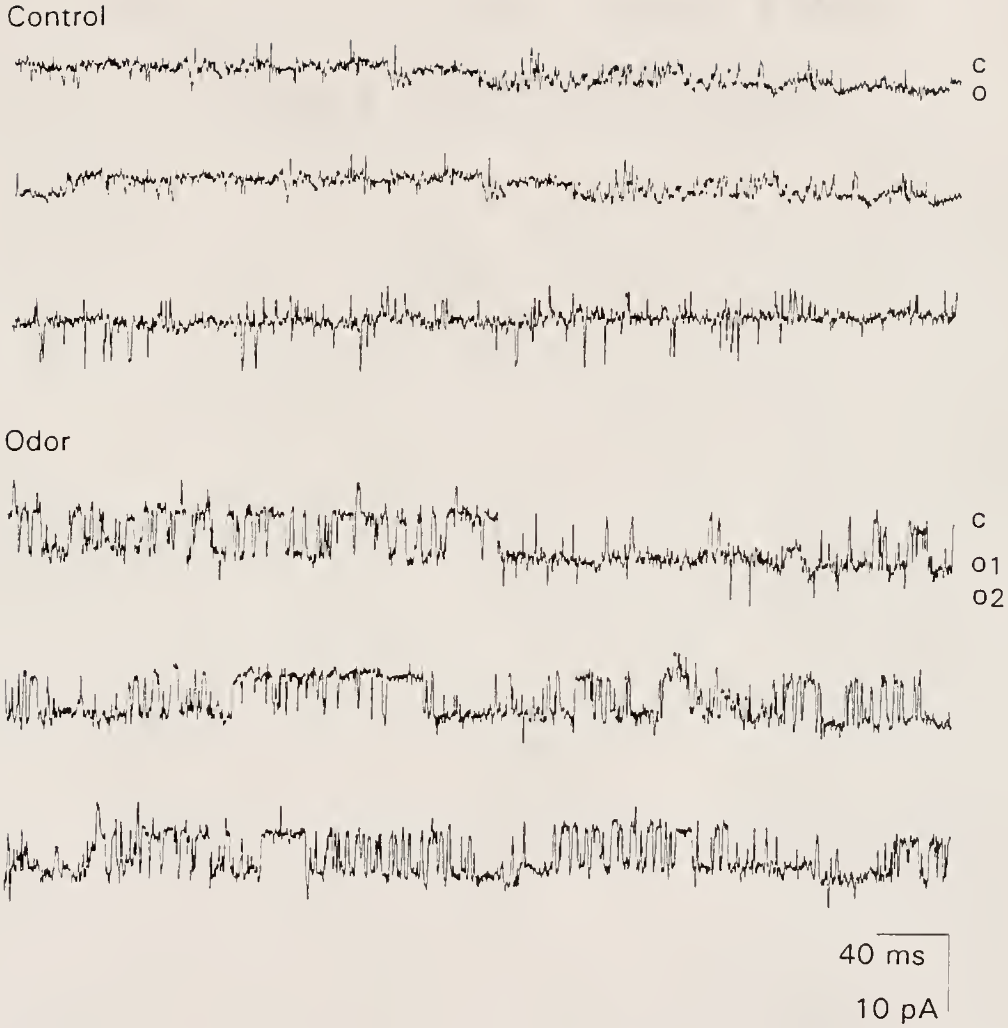
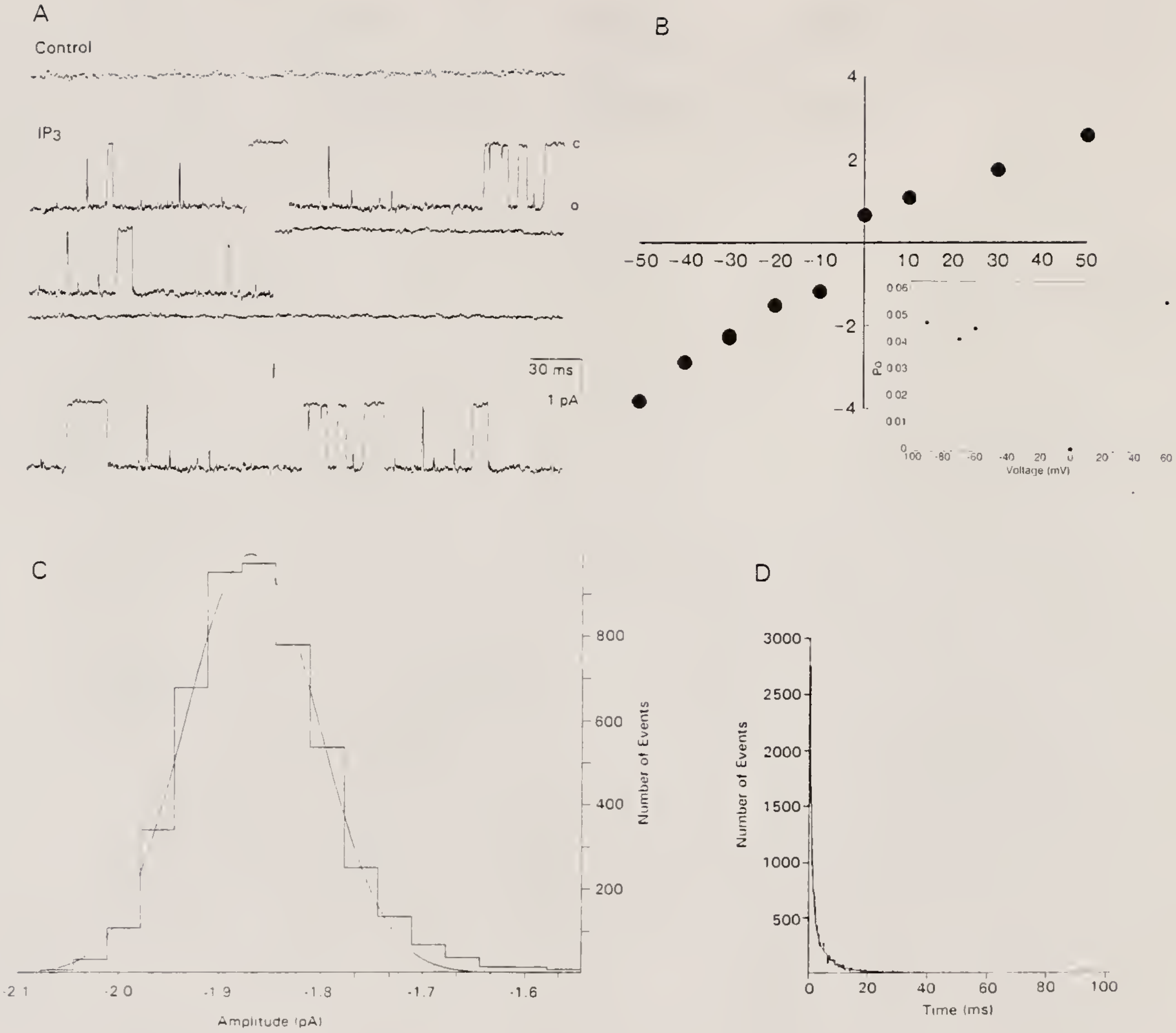

Control
c
o
Odor
c
o1
o2
40 ms
10 pA

Table 5-1: Properties of odor- and IP_3-activated channels in cultured lobster olfactory receptor neurons

Property	Large IP_3 Channel	Small IP_3 Channel	Odor Channel
Conductance	73.7 ± 5.7 pS	30.0 ± 1.6 pS	[*]86.7 ± 17.1 pS
Mean Open Time			
Tau 1	0.38 ± 0.07 msec	0.49 ± 0.06 msec	0.43 ± 0.05 msec
Tau 2	2.52 ± 0.43 msec	6.33 ± 1.64 msec	5.18 ± 1.39 msec
Mean Closed Time			
Tau 1	2.64 ± 0.19 msec	1.25 ± 0.24 msec	2.85 ± 0.37 msec
Tau 2	36.49 ± 6.80 msec	42.64 ± 15.90 msec	35.78 ± 1.34 msec

[*] Denotes estimated chord conductance rather than slope conductance. See text for sample size of each measure.

Figure 5-3. IP_3-evoked, 74 pS Unitary Currents in Inside-Out Patches of Membrane from Cultured Lobster Olfactory Receptor Neurons
A) Basal current prior to (control) and after (IP_3) "spritzing" 10^{-7} M IP_3 on the internal face of a patch. Membrane potential, -30 mV. Records filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.
B) Plot of the current-voltage relation of the channel shown in A). The current reversed near zero in symmetrical solutions with a mean slope conductance of 73.7 ± 5.7 pS (n=12). Voltage in mV on the abscissa and current in pA on the ordinate. Inset: Probability of open state (Pr_{open}) of the channel in A) at different membrane potentials.
C) Amplitude histogram of 9965 open-time events from the membrane patch in A), fit by a Gaussian distribution with mean and standard deviation of -1.87 ± 0.07 pA.
D) Plot of the open-time distribution of 2735 events of an IP_3-activated channel fit by a double exponential. τ_1 = 0.38 ± 0.07 msec, τ_2 = 2.52 ± 0.43 msec (n=14). Closed time distribution not shown.


A
Control
IP3
c
o
30 ms
1 pA
B
4
2
-2
-4
-50 -40 -30 -20 -10
10 20 30 40 50
Po
Voltage (mV)
C
800
600
400
200
Number of Events
-2.1
-2.0
-1.9
-1.8
-1.7
-1.6
Amplitude (pA)
D
3000
2500
2000
1500
1000
500
Number of Events
0
20
40
60
80
100
Time (ms)

The second type of channel (Figure 5-4A) had a mean slope conductance of 30.0 ± 1.6 pS and reversed polarity at 30.2 ± 1.5 mV in symmetrical solutions (n=16) (Figure 5-4B). The open probability function followed a Gaussian distribution (Figure 5-4C), but less closely than that of the larger conductance channel. The Pr_{open} of the channel was voltage dependent and decreased from 0.5 to 0.1 between -40 and +40 mV (Figure 5-4B, inset). The mean open (t_o) and closed (t_c) times were best fit by double exponentials (n=32)(Figure 5-4D, Table 5-1).

There was no significant difference in the distribution of t_o for the two types of IP_3-activated channels and the odor-activated channels (one-way Analysis of Variance; ANOVA). However, there was a significant difference in the distribution of t_c for the two types of IP_3-activated channels compared to that of the odor-activated channels, which could be attributed to the second exponent (τ_2) of the smaller conductance channel (ANOVA, Student-Newman-Keuls).

The Pr_{open} of both types of IP_3-activated channels (n=5, each type) increased with the concentration of IP_3 applied to the patch, as would be expected if IP_3 functioned as a second messenger. The Pr_{open} for the larger conductance channel increased from 0.04 ± 0.02 at 10^{-7} M to 0.25 ± 0.03 at 10^{-5} M, while the same parameter for the smaller conductance channel increased from 0.06 ± 0.02 at 10^{-7} M to 0.28 ± 0.13 at 10^{-5} M. Concentrations outside of this range

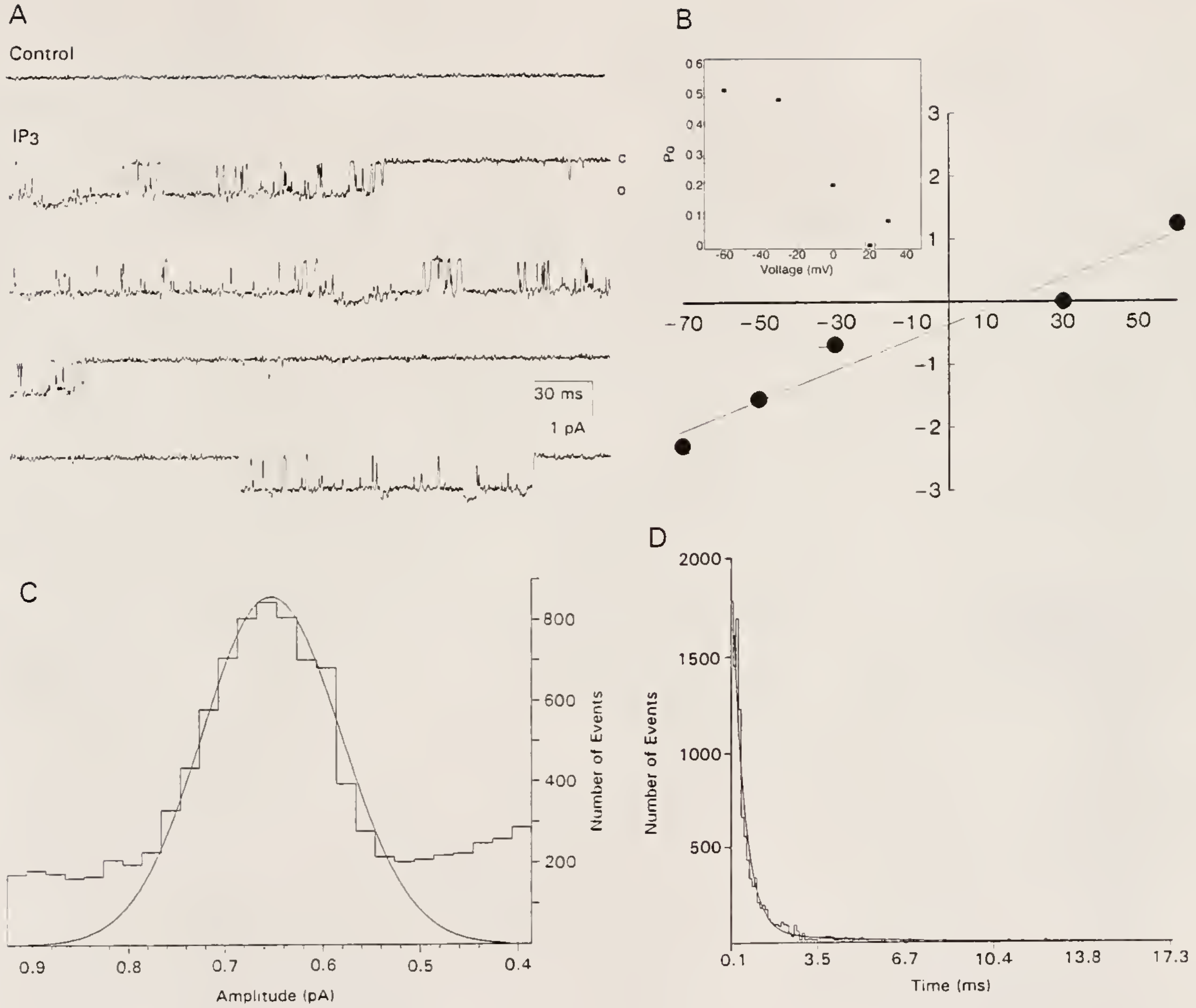
Figure 5-4. IP_3-evoked, 30 pS Unitary Currents in Inside-Out Patches of Membrane from Cultured Lobster Olfactory Receptor Neurons

A) Basal currents prior to (control) and after (IP_3) "spritzing" 10^{-7}M IP_3 on the internal face of a patch. Membrane potential, -30 mV. Records filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.

B) Plot of the current-voltage relation of the unitary currents shown in A). The current reversed between 20-30 mV in symmetrical solutions with a mean slope conductance of 30.0 ± 1.6 pS (n=16). Voltage in mV on the abscissa and current in pA on the ordinate. Inset: Probability of open state (Pr_{open}) of the channel in A) over different membrane potentials.

C) Amplitude histogram of 3699 open-time events from the membrane patch in A), fit by a Gaussian distribution with mean and standard deviation of 0.65 ± 0.02 pA.

D) Plot of the open-time distribution of 1737 events of an IP_3-activated channel fitted by a double exponential. τ_1 = 0.49 ± 0.06 msec, τ_2 = 6.33 ± 1.64 msec (n=15). Closed time distribution not shown.


A
Control
IP3
c
o
30 ms
1 pA
B
0 6
0 5
0 4
0 3
0.2
0 1
0
Po
-60
-40
-20
0
20
40
Voltage (mV)
3
2
1
-1
-2
-3
-70
-50
-30
-10
10
30
50
C
800
600
400
200
Number of Events
0.9
0.8
0.7
0.6
0.5
0.4
Amplitude (pA)
D
2000
1500
1000
500
Number of Events
0.1
3.5
6.7
10.4
13.8
17.3
Time (ms)

were not tested. When membrane patches (n=5) containing the larger conductance channel were stimulated repeatedly with 10^{-5} M IP_3, so as to continuously expose the channel to the ligand for 50 sec, the channel remained in the open state 42 ± 12% of the time and failed to desensitize. The smaller conductance channel was not tested with continuous stimulation.

Both the large (n=2) and the small (n=2) conductance channels were blocked by 10 μM RR, consistent with the pharmacology of the macroscopic current (Figure 5-5A). The blockade was partially reversible within 5 min of washout of the drug. Although not tested on the macroscopic current, both the larger (n=3) and the smaller (n=2) conductance channels were also blocked by 2.5 μM heparin, a drug known to block intracellular IP_3 receptors (Supattapone et al., 1988; Frank and Fein, 1991) (Figure 5-5B). Heparin blockade was fully reversible upon washout.

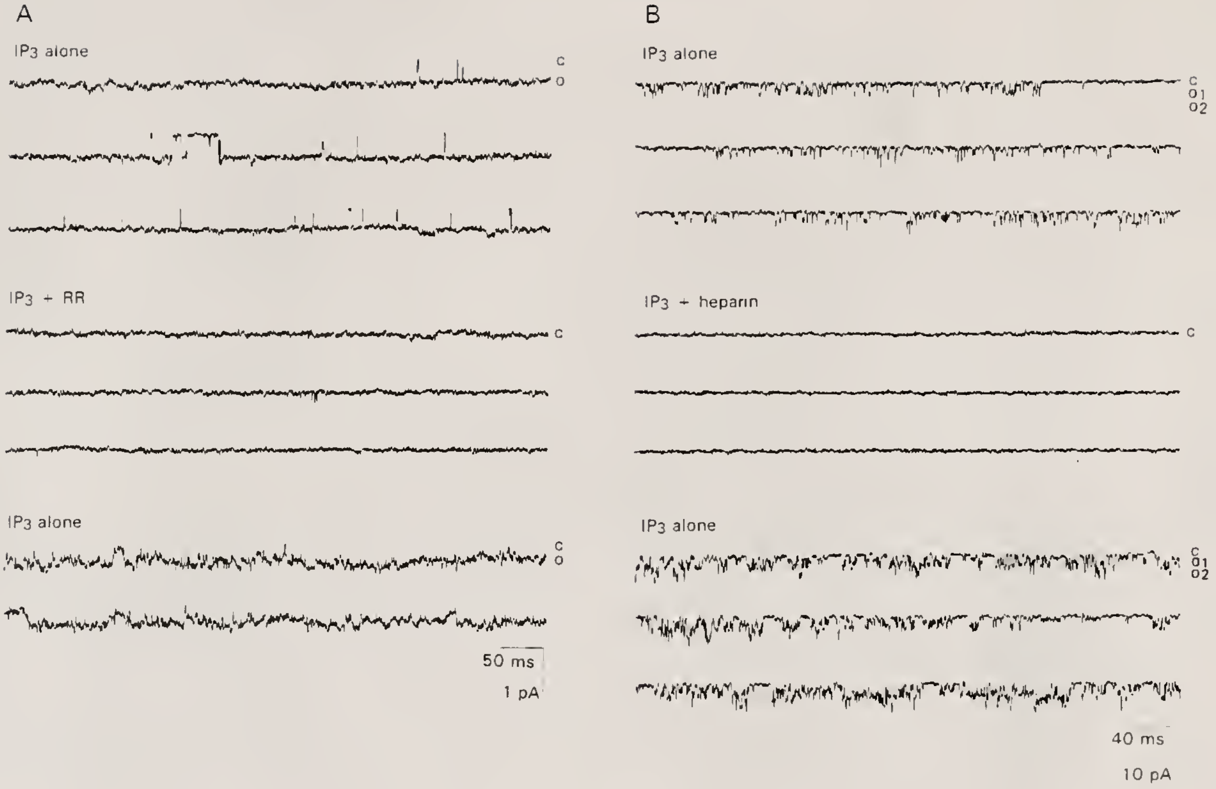
Channels of both types were insensitive to modulation by ATP, unlike the 2-4 fold increases in Pr_{open} reported for some IP_3 receptors (Ehrlich and Watras, 1988). Co-presenting up to 50 mM ATP with 10^{-7} M IP_3 failed to alter the Pr_{open} (as reported above) of either the larger (n=3) or the smaller (n=4) conductance channels (Signed-Rank Test).

In one trial, it was possible to successfully insert a pipette with an inside-out patch containing the larger conductance channel taken from one cell into a second cell

Figure 5-5. Ruthenium Red (RR) and Heparin Block of IP_3-activated Channels in Inside-Out Patches from Cultured Lobster Olfactory Receptor Neurons

A) Activity of a channel of the type shown in Fig. 5-4 induced by "spritzing" 10^{-7} M IP_3 on the inside face of the patch (upper three traces) is abolished when 10 μM RR is co-presented with IP_3 (middle three traces) and partially recovers (lower two traces) when RR is rinsed and IP_3 re-presented. Membrane potential, -60 mV. Records filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.

B) Activity of a channel of the type shown in Fig. 5-3 induced by "spritzing" 10^{-7} M IP_3 on the inside face of the patch (upper three traces) is abolished when 2.5 μM heparin is co-presented with IP_3 (middle three traces), and recovers (lower three traces) when heparin is rinsed and IP_3 re-presented. Membrane potential, -60 mV. Records were filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.


A
IP3 alone
c
o
IP3 + RR
c
IP3 alone
c
o
50 ms
1 pA
B
IP3 alone
c
o1
o2
IP3 + heparin
c
IP3 alone
c
o1
o2
40 ms
10 pA

(Figure 5-6). "Spritzing" the odor TET on the second, recipient cell induced channel activity in the patch, presumably, although not necessarily, due to elevation of intracellular IP_3, as illustrated in Figure 5-6. The activity of the channel decreased over the subsequent 24 sec.

Immunochemistry and Related Physiology

A polyclonal antibody raised against the 19 C-terminal amino acids of a cDNA clone of a mouse cerebellar IP_3 receptor (Mignery et al., 1989 - kindly supplied by Dr. P. DeCamilli; Figure 5-7), immunolabelled a band greater than 200 kDa, as well as several lower molecular weight bands (Figure 5-8A, left panel, left column). Only the immunoreactivity of the greater that 200 kDa band was incrementally blocked by preabsorbing the antibody with increasing concentrations of a synthetic peptide patterned after the original antigen PCD6 (data not shown). The antibody also labeled a band greater than 200 kDa in membranes isolated from cultured lobster ORNs (Figure 5-8A, right panel, left column). No bands were observed in either the mouse or lobster membrane preparation when the primary antibody was replaced with non-immune rabbit serum (Figure 5-8A, both panels, right columns). It was not possible to test pre-immune rabbit serum.

Introducing the antibody into the cell through the patch pipette enhanced the odor (TET)-evoked inward

Figure 5-6. Odors Activate an IP_3-gated Channel Inserted Into a Cultured Lobster Olfactory Receptor Neuron (ORN)

Left: An inside-out patch taken from one ORN was confirmed to contain an IP_3-gated channel of the type shown in Fig. 5-3 by "spritzing" 10^{-7} M IP_3 on the inner face of the patch. IP_3 was applied at the beginning of the trace shown.

Right: The patch was subsequently "crammed" into a second ORN, which was subsequently "spritzed" with TET (arrow). Application of the odor activates two superimposed channels in the patch, suggesting that the odor elevates the intracellular concentration of IP_3. The peak concentration of IP_3 in the cell is presumably greater that 10^{-7} M, since it elicited superimposed channel openings; 10^{-7} M IP_3 elicited only single channel openings in the calibration trial (left). Activity of the channels declines over the subsequent 24 sec following odor stimulation. Cartoon depicts the hypothesized mechanism of action: Odors (■) bind to cell-surface receptors and activate the enzyme phospholipase C (PLC) through G-protein (G) mediation to convert phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3), which is detected by the IP_3-gated channels in the patch. No channel activity was observed prior to odor stimulation. Membrane potential, -60 mV. Records filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.

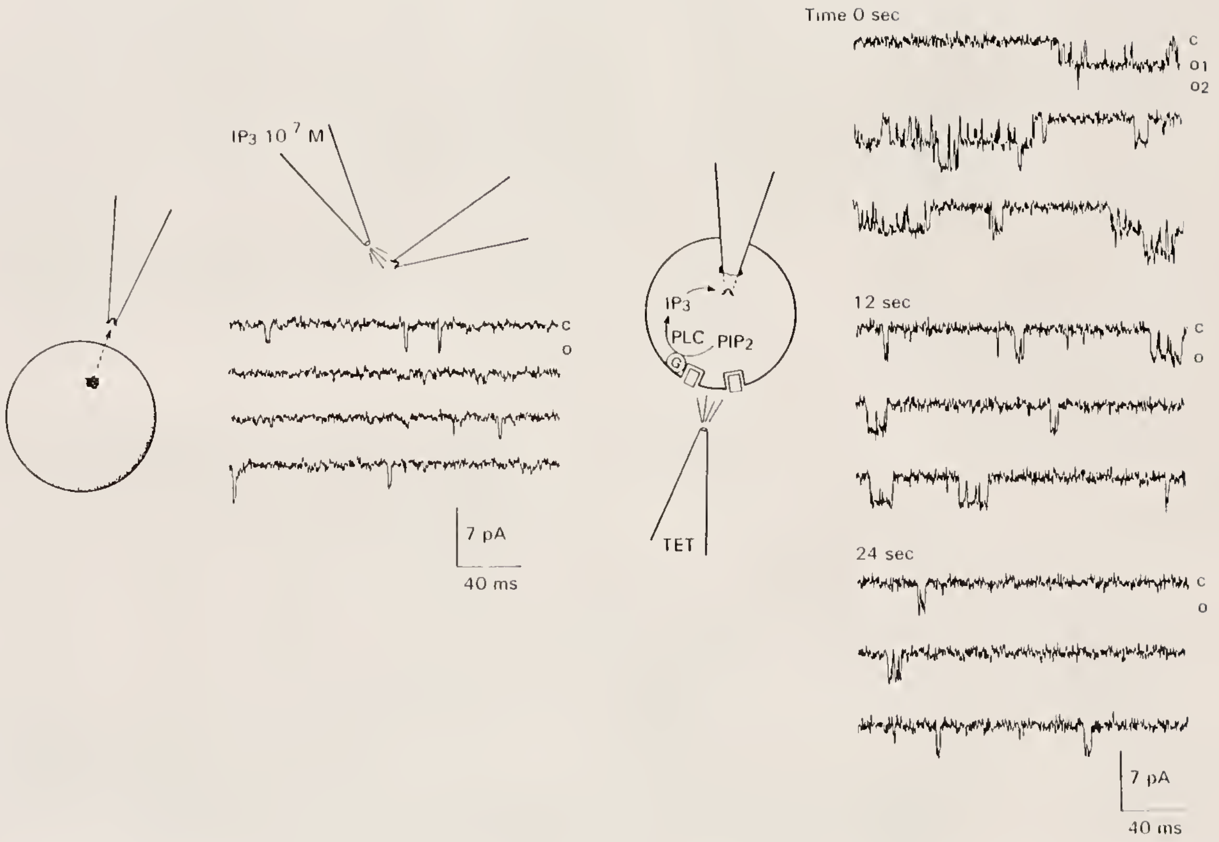
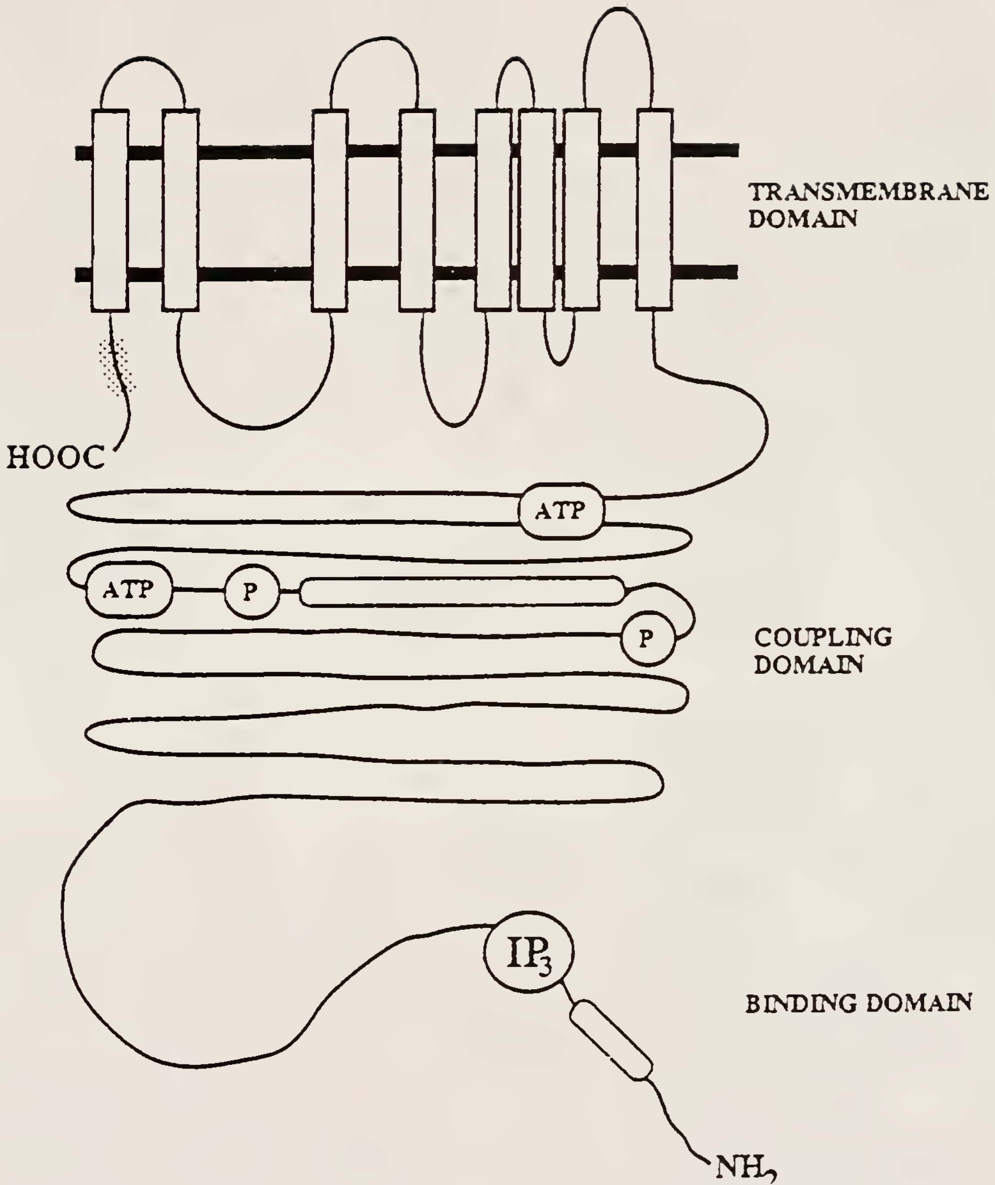

IP$_3$ 10^{7} M
c
o
7 pA
40 ms
IP$_3$
PLC
PIP$_2$
G
TET
Time 0 sec
c
o1
o2
12 sec
c
o
24 sec
c
o
7 pA
40 ms

Figure 5-7. A polyclonal antibody raised against the 19 C-terminal amino acids (shaded portion) of a cDNA clone of a mouse cerebellar IP_3 receptor was used to localize the IP_3 receptor protein in lobster by Western plot analysis and used to perturb the magnitude of macroscopic odor-evoked currents and the Pr_{open} and kinetics of IP_3-gated channels by electrophysiological analysis.

Modified, with permission from Ferris and Snyder, 1992.



from Ferris and Snyder, 1992.

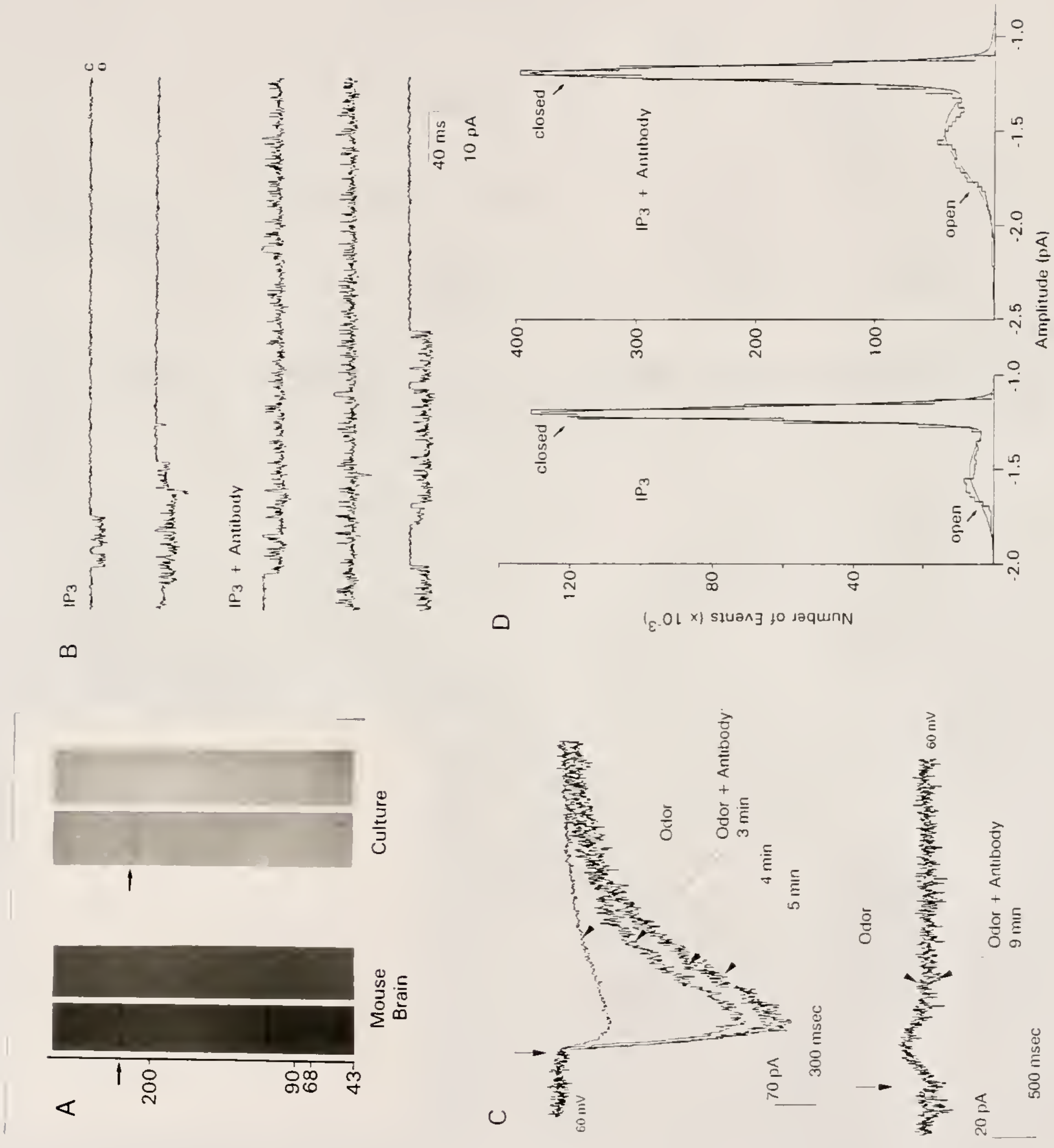
Figure 5-8. Localization and Functional Selectivity of an IP_3 Receptor in Cultured Lobster Olfactory Receptor Neurons.

A) Immunoreactivity of membrane fractions from mouse cerebellum and cultured lobster olfactory receptor neurons (culture) to an antibody directed against a cDNA clone of a mammalian IP_3 receptor (first lane of each pair) and to nonimmune rabbit IgG (second lane of each pair). Molecular weight standards as indicated. Arrow denotes antibody cross reactivity with a greater than 200 kDa protein in each preparation.

B) Upper traces: Whole-cell recording from a voltage-clamped cultured lobster olfactory receptor neuron that was sequentially patched to determine the amplitude of inward current evoked by the odor, TET (on at arrow) with (odor + antibody) and without (odor) the antibody in A) in the patch pipette. The current increased over time (as indicated) with antibody in the pipette. Membrane potential, -60 mV. Lower traces: Same protocol applied to an outward current evoked by proline (on at arrow).

C) Unitary currents recorded in an inside-out patch, activated by "spritzing" 10^{-7} M IP_3 on the inner face of the patch (IP_3) are increased when the same concentration of IP_3 is co-presented with the antibody in A) (IP_3 + Antibody). In each instance shown,the records are representative of channel activity several seconds after "spritzing". Membrane potential, -60 mV. Records filtered at 2 kHz. "C" and "O" as defined in Fig. 5-2.

D) Amplitude histogram of the unitary currents recorded in (C), activated by "spritzing" 10^{-7} M IP_3 on the inner face of the patch in the absence (IP_3) and presence (IP_3 + Antibody) of the antibody in A). The probability of open state (Pr_{open}), but not the conductance, increases in the presence of the antibody. "Closed" and "Open" state current distributions of the channel are indicated with arrows.


A
200
90
68
43
Mouse Brain
Culture
B
IP3
IP3 + Antibody
c
o
40 ms
10 pA
C
60 mV
70 pA
300 msec
Odor
Odor + Antibody
3 min
4 min
5 min
Odor
Odor + Antibody
9 min
60 mV
20 pA
500 msec
D
Number of Events (x 10^-3)
120
80
40
closed
open
IP3
-2.0
-1.5
-1.0
400
300
200
100
closed
open
IP3 + Antibody
-2.5
-2.0
-1.5
-1.0
Amplitude (pA)

current (Figure 5-8B, upper traces). The peak amplitude of the odor-evoked current increased an average of 427 ± 48% within 3 min after breakthrough over that evoked without the antibody in the pipette (5 of 5 sequentially patched cells). The effect of the antibody increased slightly over the 30 min following the second breakthrough, presumably reflecting further diffusion of the antibody from the pipette. The effect of the antibody was selective for the inward current (Figure 5-8B, lower traces). Introducing the antibody into the cell failed to visibly alter the odor (proline, taurine, alanine)-evoked outward current (9 of 9 sequentially patched cells). Without antibody in the pipette, odor (TET, proline, taurine, betaine)-evoked currents of both polarities decreased up to 43% of their initial magnitude over the 20-30 min following breakthrough. To determine if the effect on the inward current was specific for the antibody, another group of cells was single-patched with pipettes that were tip-filled with normal patch solution and backfilled with either heat inactivated antibody (8 cells), rabbit serum (7 cells) or an antibody raised against G_{olf} (16 cells; a generous gift of R. Bruch; this antibody does not recognize any protein in lobster ORNs by Western blot analysis - unpublished data), and the protein allowed to diffuse into the cell for up to 30 min. All three control proteins failed to visibly alter the magnitude of the odor-evoked current of either polarity from that observed without antibody in the pipette (arcsin transformation of percentage data followed by Student's t-test). Using this same technique, but backfilling with the antibody enhances the odor (TET)-evoked inward current to approximately the same extent observed in the sequentially patched cells (375 ± 29 %, 4 cells), suggesting that the control proteins were able to diffuse into the cells during the experimental interval. In the absence of odor stimulation, the antibody had no effect on the basal current (data not shown).

The antibody increased the ability of 10^{-7} M IP_3 to activate unitary currents in 4 of 5 cell-free patches (Figure 5-8C). The antibody increased the Pr_{open} of all channels in the four patches (n = 2 small and 5 large) from an average of 0.11 ± 0.06 to 0.41 ± 0.07 (Figure 5-8D). In one patch that contained multiple channels (4 large), the channels showed a marked increase in superposition in the presence of the antibody; from 1-2 of the channels being open 78% of the time to 3-4 of the channels being open 65% of the time. The antibody selectively increased the duration of the second exponent (τ_2) of the mean open time (t_o) from 0.84 ± 0.17 msec to 19.06 ± 9.66 msec; the other kinetic parameters and the conductance were unaltered (n=4). In the fifth patch, the antibody first decreased, and then blocked, the activity of a large conductance channel.

Discussion

The ability of odors to increase channel activity in cell-attached recordings argues strongly for the involvement of a diffusible second messenger in the excitatory transduction pathway. Second messenger mediation is also consistent with evidence that the odor-evoked inward current in lobster ORNs is affected by probes directed against GTP-dependent proteins (see Chapter 4) that presumably would link receptor activation to second messenger production.

Several findings argue that IP_3 is the excitatory second messenger. (1) IP_3 selectively evokes macroscopic inward currents, which would be expected to depolarize (excite) the cells. (2) RR blocks both the odor-evoked macroscopic current and the IP_3-gated unitary current. (3) An odor (TET) activates an IP_3-gated channel inserted into the cell, as would be expected if odor-binding elevated the intracellular concentration of IP_3. Although, in the latter instance, I cannot exclude that the odor altered the intracellular environment in some way other than elevating IP_3, preliminary findings suggest that altering intracellular pH from 5-9 and elevating $[Ca^{2+}]_i$ up to 30 mM fails to initiate channel activity in cell-free patches.

The ability of IP_3 to elicit channel activity in cell-free patches argues that IP_3 acts directly on the channels and not through activation of a protein kinase or some other additional step in the transduction cascade, since any soluble enzymes or substrates required for activation would presumably be diluted below threshold concentrations in the bath shortly after patch excision. I cannot conclude directly that the IP_3-gated channels observed in cell-free patches are the same as the channels activated by odors in cell-attached recordings. My finding that the unitary currents in both recording configurations have similar mean open times and that both the odor-evoked macroscopic current and the IP_3-gated channels observed in cell-free patches can be blocked by RR argues that the IP_3-gated channels observed in cell-free patches are a component of an IP_3-mediated excitatory transduction cascade.

Finding channels on the soma of cultured ORNs that presumably function on the dendritic processes of these cells *in situ*, would be consistent with the finding that cAMP-gated channels that mediate excitation in the cilia of dissociated vertebrate ORNs also occur in low density on the dendrite and soma (Nakamura and Gold, 1987; Firestein et al., 1991). Whether the IP_3-activated channels normally occur on the soma of lobster ORNs or are inserted there prior to relocation to the dendritic processes during neurite outgrowth in culture remains to be tested.

I conclude that IP_3 gates two different channels rather than one channel with two different sub-conductance states, as has been reported for IP_3-activated channels in canine cerebellum or aortic smooth muscle (Mayrleitner et al., 1991; Watras et al., 1991). Save for one patch, the large and small conductance channels occurred in different patches of membrane. Differences in the voltage dependencies and reversal potentials in symmetrical ionic conditions further support the conclusion that IP_3 gates two different channels. Since two variably spliced mRNAs code for intracellular IP_3 receptors (e.g., mouse cerebellar IP_3 receptor - Nakagawa et al. 1991), it would not be inconsistent to have two subtypes of IP_3-activated channels expressed in lobster ORNs.

The lack of identical recording conditions makes it difficult to compare the lobster olfactory channels with other reported IP_3-activated channels based on their respective conductances (Enrich and Watras, 1988; Restrepo et al., 1990; Maeda et al., 1991; Mayrleitner et al., 1991), but the ability of RR and heparin to block the IP_3-gated channels in lobster ORNs is similar to the pharmacological properties of intracellular IP_3 receptors. RR inhibits IP_3-activated channels in skeletal muscle SR but not in aortic smooth muscle (Enrich and Watras, 1988). Heparin, which is thought to block the IP_3-binding site (Supattapone et al., 1988), acts in a variety of cell types (Hill et al., 1987; Worley et al, 1987; Ghosh et al., 1988; Kobayashi et al., 1988; Komori and Bolton, 1990; Frank and Fein, 1991; Fisher et al., 1992). The reversibility of the heparin block that we observed with washing has also been reported in smooth muscle (Mayrleitner et al., 1991) and demonstrates that heparin, which is known to be cytotoxic at high concentrations (Frank and Fein, 1991), did not damage the membrane.

The IP_3-activated channels in lobster ORNs differ from intracellular IP_3 receptors in their ATP dependency. While ATP failed to alter the conductance or the mean open time of the channels in lobster ORNs, it increases IP_3-induced macroscopic currents in aortic smooth muscle SR (Mayrleitner et al., 1991) and the Pr_{open} of intracellular IP_3-gated channels (Ehrich and Watras, 1988; Mayrleitner et al., 1991). The ATP-driven Ca^{2+} pump postulated to be associated with the IP_3-release mechanism in other, non-sensory cells (Ferris et al., 1990) may not be functional in sensory transduction and therefore not present or active in the lobster cells. Danoff et al. (1991) report mRNA coding for IP_3 receptors in rat and human that lack the SII region of the channel protein that contains the ATP binding sites. From my electrophysiological data alone, I cannot assign the apparent lack of modulation by ATP to protein variation, species differences or olfaction.

The IP_3-activated channels in lobster ORNs presumably share some structural homology with mammalian IP_3 receptors, since an antibody raised against a cDNA clone of a mammalian IP_3 receptor recognized a protein of similar size in the lobster. The molecular weight (>200K) of the immunolabelled band in lobster ORNs is consistent with the size of intracellular IP_3 receptors, that are proposed to be a tetramer of noncovalently bound isomers with a characteristic M_r of 260K by SDS-PAGE analysis (Mignery et al., 1989; Fisher et al., 1992). That the antibody is binding to a functionally relevant molecule is suggested by the ability of the antibody to selectively increase odor-evoked inward currents. The ability of the antibody to selectively increase the Pr_{open} and t_o of IP_3-activated channels argues further that the antibody targets the channel to augment the macroscopic current. The antibody would not necessarily be expected to target the transmembrane pore and block ion flow, as it was directed against the 19-amino acid carboxyl terminus of the receptor that DeCamilli et al. (1990) postulate is involved in Ca^{2+} sequestration. Indeed, a monoclonal antibody targeting a different epitope of the C-terminus has been shown recently to block IP_3- and Ca^{2+}-induced release of Ca^{2+} in fertilized hamster eggs, suggesting that the C-terminus can regulate channel gating (Miyazaki et al., 1992). Independent of mechanism, the ability of the antibody to selectively enhance odor-evoked inward currents independently of outward currents provides further support that IP_3 is the excitatory second messenger.

Thus, from the data presented in this chapter, I conclude that the IP_3 receptors in cultured lobster ORNs are in the plasma membrane since (1) a high percentage of cell-free patches (63 of 86 successful seals) taken from the plasma membrane are directly activated by IP_3 and (2) an antibody directed against a known IP_3 receptor alters IP_3-activated unitary currents in cell-free patches of plasma membrane. It is not likely that organelles closely apposed to the plasma membrane would be frequently drawn into the patch. Even if this occurred, the plasma membrane would need to be disrupted in order to reseal onto the organelle, and the organelle membrane would need to be recorded in the outside-out configuration for IP_3 to bind. Earlier reports differ on whether IP_3 receptors occur in the plasma membrane of neurons. In studies of IP_3 receptors in the cerebellum, Maeda et al. (1989) localized the P400 (IP_3) receptor to the ER, postsynaptic densities and the plasma membrane, while Ross et al. (1989) localized the receptor to the ER, subplasmalemmal cisternae, and nuclear membrane, and NOT to the plasma membrane. Immunogold labeling with the same antibody used in this report failed to reveal an IP_3 receptor in the plasma membrane of cerebellar neurons (Mignery et al., 1989). Yet, my conclusion is consistent with evidence emerging from studies of olfactory neurons. Khan et al. (1992) identified a 260 kDa protein in the plasma membrane of rat olfactory cilia that stained with antiserum to a cerebellar IP_3 receptor in Western blots. Kalinoski et al. (1992) photoaffinity labeled a 107 kDa protein in the plasma membrane of catfish olfactory cilia which binds IP_3. Restrepo et al. (1990) were able to reconstitute an IP_3-activated channel from a membrane preparation of catfish olfactory cilia. To the extent that the membrane preparations used in these studies are purely ciliary, these findings argue for plasma membrane receptors since the cilia of ORNs are devoid of organelles. Indeed, Cunningham et al. (1992) have preliminary evidence that an immunogold labelled antibody directed against a cerebellar IP_3 receptor labels rat ciliary membrane. The plasma membrane IP_3 receptors in olfactory receptor cells may represent a new class of IP_3 receptors that share structural homology with intracellular IP_3 receptors, but are localized to the plasma membrane.

My ability to record IP_3-activated unitary currents in native plasma membrane of lobster ORNs and to tie these unitary currents to odor-activated macroscopic inward currents directly implicates plasma membrane IP_3 receptors in olfactory transduction. I thereby conclude that odors excite lobster ORNs by increasing intracellular IP_3, which activates IP_3-gated channels in the plasma membrane of the cell and depolarizes the cell in a concentration dependent manner. The IP_3-mediated transduction pathway presumably co-exists in some cells with a second, cAMP-mediated pathway that hyperpolarizes the cell and modulates the magnitude of excitation to reflect the particular ratio of excitatory and inhibitory odor components in an odor blend (Michel et al., 1991; Michel and Ache, in press). Having two, parallel transduction pathways, one mediated by IP_3 and the other by cAMP, allows the lobster ORN to serve as an integrating unit. The ability of olfactory receptor cells with parallel transduction pathways to integrate information about the composition of odor blends may be integral to odor coding, as suggested by Reed (1992), and may explain why odors activate both IP_3- and cAMP-mediated second messenger pathways in animals as phylogenetically diverse as arthropods, fish and mammals, as noted in the Introduction.

Experimental Procedures

Solutions

Panulirus saline (PS) consisted of (in mM): 458 NaCl, 13.4 KCL, 9.8 $MgCl_2$, 13.6 $CaCl_2$, 13.6 Na_2SO_4, 3 HEPES, and 2 glucose; pH 7.4. Modified L15 Media consisted of: 50 ml Liebowitz L15 stock, 50 ml of 1.6 times the normal concentration of PS, 0.6 g dextrose, 0.026 g L-glutamine, and 0.01% gentamicin. Phosphate-sucrose buffer (PSB) consisted of (mM): 10 Na_2P0_4 and 250 sucrose; pH 7.3. Homogenization buffer (HB) consisted of (mM): 320 sucrose, 10 Tris base, 50 KCl, and 1 EDTA. Tris buffered saline (TBS) consisted of (mM): 50 Tris HCl, 150 NaCl; pH 7.5. Tween-TBS (TTBS) was made by adding 0.1% Tween-20 to TBS. Blotting buffer (BB) consisted of (mM): 25 Tris HCl, 192 glycine, plus 0.1% SDS and 10% methanol. Normal patch solution consisted of (mM): 30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 180 K-acetate, and 696 glucose; pH 7.0. Ruthenium red (10 μm) and heparin (2.5 μm) were prepared fresh daily.

Odors were an aqueous extract of TetraMarin, a commercial flake fish food (Tetra Werke, Melle, FRG) and solutions of d-alanine, betaine, glycine, l-proline and taurine; compounds known to be adequate stimuli for the chemoreceptors of aquatic organisms such as lobsters (Carr, 1988). A stock extract of TetraMarin (TET) was made by mixing 2 gm dry flakes in 60 ml saline, centrifuging the resulting suspension at 1400 g, filtering it through Whatman #3 filter paper, adjusting it to pH 7.4, and storing it frozen in 5 ml aliquots. The stock extract was thawed and diluted 1000-fold in either PS or culture media, as appropriate, just prior to use and applied at that concentration. The pure compounds were prepared fresh as 10^{-3} M solutions in either PS or culture media, as appropriate, adjusted to pH 7.4, as needed, and applied at that concentration.

All drugs and chemicals were obtained from Sigma Chemical Co, except for the culture media. Sources for the culture media and supplements are listed in Fadool et al. (1991b).

Tissue Culture

Distinct clusters of ORNs were dissected from the olfactory organs (lateral antennular filaments) of adult specimens of the Caribbean spiny lobster, *Panulirus argus*, and enzymatically dissociated. The resulting cells were sustained in primary culture as described previously (see Chapter 2). Briefly, the isolated clusters were incubated for 50 min at 80 rpm on an orbital shaker in 0.2 micron filter-sterilized solution of 2.5 mg papain and 12 mg L-cysteine in 10 ml *Panulirus* saline (PS) containing 1% penicillin, streptomycin sulfate, and amphotericin B (Gibco). Proteolytic digestion was stopped by replacing the enzyme solution with low glucose L-15 media supplemented with L-glutamine, dextrose, fetal calf serum, and BME vitamins. Cells were immediately plated on poly-d-lysine-coated glass coverslips. Cells were maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) at 24°C.

Electrophysiology

The cultured cells were viewed at 40X magnification with Hoffman optics for patch-clamp recording (Hamill et al., 1981). Patch electrodes, fabricated from 1.8 mm O.D. borosilicate glass and fire polished to a tip diameter of approximately 1.0 μm (bubble number 4.8; Mittman et al., 1987), produced seal resistances between 8 and 14 GΩ. The electrodes were uncoated. Signals were amplified with an integrating patch amplifier (Dagan 3900).

Macroscopic currents were recorded in the whole-cell configuration at a holding potential of -60 mV, unless noted otherwise, using normal patch solution in the pipette. The analog records were filtered at 5 kHz and digitally sampled every 4 msec (every 240 msec, in one noted instance) for subsequent processing on an IBM-compatible computer using pCLAMP software (Axon Instruments). The compact, spherical shape of the cultured cells (see Chapter 2) facilitated obtaining an effective space clamp on most cells. Odors and membrane permeant probes were "spritzed" on the cells for 120 msec from a six barrel glass micropipette coupled to a pressurized valve system (Picospritzer, General Valve). The concentration of odors and probes delivered in this manner are reported as the pipette concentration; no attempt was made to correct for dilution. In order to compare the effect of impermeant probes on cell activity, cells were sequentially patched, first without, then with the probe in the pipette in order to provide a within-cell control. There was no noticeable effect on the response of sequentially patched cells to odor or voltage stimulation in the absence of any experimental manipulation.

Unitary currents were recorded in either the cell-attached or (primarily) the cell-free, inside-out configuration. The normal patch pipette solution was a potassium acetate patch solution (see solutions); both faces of the patch were exposed to symmetrical solutions. The analog records were filtered at 2 kHz and recorded on videotape. On playback, the records were sampled every

100 μsec for processing on an IBM-compatible computer, also using pCLAMP software (Axon Instruments). Probes were "spritzed" on the inner face of the patch for 550 msec from the multibarreled pipette mentioned above.

All results are presented as the mean ± standard error of the mean. Statistical significance was calculated at the 95% confidence level.

Immunochemistry

Mouse cerebellum was isolated then diced in chilled phosphate sucrose buffer (PSB). Thirty-six hour cultured ORNs were scraped with a rubber policeman, centrifuged below 1,000 RPM for 2 min at 4°C, and removed from media without disrupting the cells. Both tissues were individually homogenized in chilled homogenization buffer (HB), either with 50 strokes by mortar and pestle (Wheaton, Size B) or 2 min by a Kontes tissue grinder in microcentrifuge tubes, and then tip sonicated (Heat systems, Ultrasonics Inc., W-220) three times for 15 s at a #3 setting while iced. Homogenates were centrifuged twice at 5,000 RPM for 30 min at 4°C. The respective supernatants were combined and centrifuged (Beckman L70 Ultracentrifuge) at 38,000 RPM for 2.5 h at 4°C. The recovered pellets were resuspended in HB by bath sonification (Heat systems, Ultrasonics Inc., W-225) three times for 15s at a #5 setting and frozen at -80 °C until use.

Protein was determined by a Bradford photometric assay. Twenty to twenty-five micrograms of the membrane preparation was separated at constant current (25 mA; 3 h) on a 5-15% gradient SDS electrophoretic gel (0.55 mm, 29:1 acrylamide/bis-acrylamide). Proteins were transferred to nitrocellulose in a modified blotting buffer (BB) at 0.5 mA for 3.5 h. Nitrocellulose membrane was soaked twice for 10 min in Tween-tris buffered saline (TTBS). Non-specific binding was blocked by incubating the membrane for 30 min with 2.5% Carnation dry milk in TTBS. The nitrocellulose membrane was then incubated for 15 h at 4°C with a polyclonal antibody (1:200) raised against the 19 C-terminal amino acids of a cDNA clone of a mouse cerebellar IP_3 receptor (Mignery et al., 1989; Figure 5-7), washed twice for 10 min in TTBS to remove unbound antibody, and reincubated for 2 h with a 1:250 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim). Gels were visualized with 4-chloro-1-naphthol color reagent activated by 30% H_2O_2 in tris buffered saline (TBS) and 20% methanol.

In control experiments, membrane preparations (750 μg total protein) were separated with a continuous front, 8% gradient SDS electrophoretic gel. Proteins were transferred to nitrocellulose, as previously, but were subsequently incubated with preabsorbed antibody. The antibody was preabsorbed by incubating the antibody for 30 min on ice with 10:1, 1:1, 0.1:1, 0.01:1, and 0:1 molar ratios of the peptide PCD6 (Mignery et al., 1989; the last 19C terminal amino acids) to antibody. Following incubation, the samples were centrifuged below 1,000 RPM for 30 min at 4°C to remove particulates and antibody-peptide complexes. The respective supernatants were applied as 200 μl samples using a miniblotter.

CHAPTER 6
ION SELECTIVITY AND MODULATION OF IP3-ACTIVATED CHANNELS

Introduction

Across a broad range of species, odor-evoked second messenger molecules (Breer, Boekhoff, and Tareilus, 1990; Ronnett, Cho, Hester, Wood, and Snyder, 1993; Ache, Hatt, Breer, and Zufall, in press) directly gate ion channels in olfactory receptor neurons (ORNs) to create a change in membrane potential (Nakamura and Gold, 1987; Suzuki, 1990; Restrepo, Miyamoto, Bryant, and Teeter, 1990; Firestein, Zufall, and Shepherd, 1991; Kurahahi and Kaneko, 1991; Zufall, Firestein, and Shepherd, 1991; Fadool and Ache, 1992a). Recent evidence strongly supports the inositol phosphate pathway in olfactory transduction, in addition to the earlier supported role of cyclic nucleotides (Reviews: Firestein, 1992; Reed, 1992; Ronnett and Snyder, 1992). Odors rapidly and transiently elevate levels of $InsP_3$ in olfactory cilia of catfish (Fitzgerald, Restrepo, and Bryant, in press) and rats (Breer, et al., 1990) and in the outer dendrites of lobsters (Ache et al., in press);

NOTE: This chapter will be submitted to J. Gen. Physiology and is formatted to meet its style regulations.

functionally equivalent structures that are the site of transduction in olfactory receptor cells (Nakamura and Gold, 1987; Kurahashi, 1989; Firestein et al., 1990). $InsP_3$ receptors can be reconstituted from olfactory cilia in catfish (Restrepo et al., 1990) and immunocyto-chemically localized to these same structures in rats (Cunningham, Reed, Ryugo, Snyder, and Ronnett, 1992). Known blockers of $InsP_3$-gated channels in other systems block odor-evoked currents in olfactory receptor cells in catfish and lobster (Restrepo et al., 1990; Fadool and Ache, 1992a).

An olfactory specific $InsP_3$-receptor has not yet been cloned, but $InsP_3$ receptors involved in olfactory transduction clearly differ from cerebellar $InsP_3$ receptors mediating Ca^{2+} mobilization in the endoplasmic reticulum (Review: Berridge, 1993). Most obvious is that olfactory $InsP_3$ receptors are plasma membrane receptors (Cunningham et al., 1992; Fadool and Ache, 1992a; Khan, Steinter, Snyder, 1992). The olfactory $InsP_3$ receptor in catfish binds $InsP_3$ and $InsP_4$ with equal affinity (Kalinoski, Aldinger, Boyle, Huque, Marecek, Prestwich, and Restrepo, 1992) in contrast to the specificity of the cerebellar $InsP_3$ receptor which binds other metabolites, including $InsP_4$, only at high concentrations and with low affinity (e.g. Ferris, Huganir, Supattapone, and Snyder, 1989). Functionally, however, olfactory $InsP_3$ channels in catfish, rat, and lobsters are selectively gated by $InsP_3$ (Restrepo et al., 1990; Restrepo, Teeter, Honda, Boyle, Marecek, Prestwich, and Kalinoski, 1992; Fadool and Ache, 1992a, 1993b). Unlike cerebellar $InsP_3$ receptors (Ferris, Huganir, and Snyder, 1990; Maeda, Kawasaki, Nakade, Yokota, Taguchi, Kasai, and Mikoshiba, 1991; Watras, Bezprozvanny, and Ehrlich, 1991), olfactory $InsP_3$-activated channels are not modulated by ATP (Fadool and Ache, 1992b), are blocked by both heparin and ruthenium red (RR) (Restrepo et al., 1990; Fadool and Ache, 1992a), and do not appear to have multiple conductance states (Fadool and Ache, 1992a). Certain transduction elements have been cloned and shown to be expressed in a highly olfactory specific manner making the likelihood of an olfactory specific $InsP_3$ receptor highly probable. Examples of such elements include the G-protein, G_{olf} (Jones and Reed, 1989; Jones, 1990; Jones, Masters, Bourne, and Reed, 1990) or the olfactory cyclic nucleotide gated channel (Dhallan, Yau, Schrader, and Reed, 1990; Ludwig, Margalit, Eisman, Lancet, and Kaupp, 1990; Goulding, Ngai, Kramer, Colicos, Axel, Siegelbaum, and Chess, 1992).

There are two different $InsP_3$ channels in the soma membrane of cultured lobster ORNs, both of which potentially are involved in olfactory transduction (Chapter 5). Lobster ORNs in culture appear to express the elements of the transduction cascade in the soma (Chapters 2-5). These channels, which only rarely occur in the same patch of membrane, differ in mean conductance (30 pS and 74 pS), voltage sensitivity, and mean closed time (t_c) kinetics, but have similar pharmacology to RR and heparin, concentration dependency, and similar altered gating when bound to an antibody directed against the carboxyl terminus of a cerebellar $InsP_3$ receptor (Chapter 5). In this chapter I further characterized the biophysical properties of the two types of $InsP_3$-gated channels in cultured lobster ORNs to better understand any functional difference between the two olfactory $InsP_3$-gated channels, and the role of the inositol phosphate pathway in olfactory transduction in general.

The data demonstrate that the large (74 pS)- and small (30 pS)-conductance $InsP_3$-gated channel respond similarly to increased Ca^{2+} or pH; have analogous long open modal gating patterns; comparable pharmacology with respect to TTX, TEA, ruthenium red, and Co/Cd; and nearly identical ionic selectivity, predominantly that of non-selective cation channels. This chapter provides a comparison of the olfactory $InsP_3$-gated channels with the cerebellar $InsP_3$-gated channels. Speculation as to significance of the apparent redundancy in having more than one channel gated by the same second messenger is offered in the discussion.

Materials and Methods

Solutions

The compositions of *Panulirus* saline (PS), and patch electrode (PE) and bath solutions are listed in Table 6-1. Modified L15 Media consisted of: 50 ml Liebowitz L15 media,

(in mM)	NaCl	EGTA	HEPES	$CaCl_2$	CH_3COOK	KCl	$BaCl_2$	$MgCl_2$	$CsCl_2$	$ChCl_2$	Glucose
Control KA Patch	30	11	10	1	180						696
Ca Patches	30		10	0.001 to 30	180						595 to 699
Ba Patch			10		180		30				655
Panulirus saline (PS)	460		10	13		13		10			
Na IP_3 Bath	480		20	0.1							1.7
Na100Cs Bath	380		20	0.1					100		1.7
Ca Bath			10	60						420	

50 ml of 1.6X normal concentration of PS, 0.6g dextrose, 0.026 g L-glutamine, and 0.01% gentamicin. Tetrodotoxin (TTX, 1 μM) as well as all control and modified $InsP_3$ solutions were made up in PE as concentrated stock solutions and stored frozen at -20°C until use. Single aliquots of $InsP_3$ were thawed and diluted in PE to a working concentration of 2.4×10^{-7} M. Care was taken during storage and use of $InsP_3$ to minimize its exposure to light, as its sensitivity to degradation is suspected. pH-modified $InsP_3$ solutions are estimates to within ± 0.5 pH unit based upon litmus paper reaction. Ten micromolar ruthenium red (RR) was prepared by grinding 0.55 mg (MW≈551) of RR (≈38% dye content) into 10 ml PS until visibly reconstituted. Solution was then filtered (0.2 μm) to remove any particulates. As the precise dye content, molecular weight, and solubility of this product are unknown, 10 μM RR represents an upper limit estimate of concentration. Thapsiagargin was stored in a concentrated EtOH solution at -20°C until use, and then diluted with PS to a working concentration of 8×10^{-7} M. Tetraethylammonium (TEA, 5 mM) and Co/Cd (5 mM each) were made as needed in either culture media or PE as appropriate for whole-cell or single-channel recording, respectively.

Odorants consisted of either single components (10^{-3} M) made daily in PS: taurine, betaine, l-proline, l-arginine, or l-glycine, AMP, or a complex odorant mixture, TetraMarin

(TET), a marine fish food. A stock extract of TET was made by mixing 2 gm dry flakes in 60 ml saline, centrifuging the resulting suspension at 1400 g, filtering it through Whatman #3 filter paper, adjusting it to pH 7.4, and storing it frozen in 5 ml aliquots. The stock was thawed and diluted 1:1000 in either culture media or PE as appropriate for whole-cell or single-channel recording, respectively.

Sources for the culture media and supplements are listed in Chapter 2. All salts, amino acids, TEA, Co/Cd, RR, and $InsP_3$ were obtained from Sigma Chemical Company. Thapsiagargin was purchased from Calbiochem.

Animals

Adult specimens of the Caribbean spiny lobster, *Panulirus argus*, were collected in the Florida Keys and maintained in the laboratory in a running seawater. Animals were fed a mixed diet of frozen fish, squid, and shrimp.

Tissue Culture

The distinct clusters of olfactory (aesthetasc) receptor neurons (ORNs) were dissected from the olfactory organ (lateral antennular filament) of adult specimens of the Caribbean spiny lobster, *Panulirus argus*. The clusters were enzymatically dissociated, and the resulting cells sustained in primary culture as described previously (Chapters 2-5). Briefly, the isolated clusters were incubated for 50 min at 80 rpm on an orbital shaker in 0.2 micron filter-sterilized solution of 2.5 mg papain and

12 mg L-cysteine in 10 ml *Panulirus* saline (PS) containing 1% penicillin, streptomycin sulfate, and amphotericin B (Gibco). Proteolytic digestion was stopped by replacing the enzyme solution with low glucose L-15 media supplemented with L-glutamine, dextrose, fetal calf serum, and BME vitamins. Cells were immediately plated on poly-d-lysine-coated glass coverslips. Cells were maintained at saturation humidity in a modular incubator chamber (Billups-Rothenberg) at 24°C.

Electrophysiology

Patch electrodes were fabricated from 1.8 mm O.D. borosilicate glass and fire polished to a tip diameter of approximately 1.0 μm (bubble number 4.8; Mittman, Flaming, Copenhagen, and Belgum, 1987). High resistance seals of between 8 and 14 GΩ were obtained. Cells were viewed at 40X magnification with Hoffman optics.

Unitary currents were recorded from cell-free patches of the soma plasma membrane in the inside-out configuration. All patches were voltage-clamped at -60 mV holding potential, unless noted otherwise. The unitary currents were recorded with an integrating patch-clamp amplifier (Dagan 3900), filtered at 2 kHz, and captured on videotape. On playback, the analog records were sampled every 100 μsec for processing on an IBM-compatible computer, using pCLAMP software (Axon Instruments). $InsP_3$ solutions were "spritzed" on the inner face of the patch for 550 msec from a seven barrel glass micropipette (Frederick haer) coupled to a single pressurized valve system (Picospritzer, General Valve) via a 6-way rotary valve as previously described (Chapter 2). The normal patch pipette solution was a potassium acetate patch solution (KA patch, Table 1); both faces of the patch were exposed to symmetrical solutions unless noted otherwise.

Odor-activated macroscopic currents were recorded by patch-clamping the ORNs in the whole-cell configuration and were also held at -60 mV membrane potential. Odorants and membrane permeant pharmacological probes were "spritzed" for 120 msec, as described, with the exception of the Ca^{2+}-ATPase pump inhibitor, thapsiagargin, which was applied to the culture medium 2 hours prior to patch-clamp recording. Macroscopic currents were filtered at 10 kHz and sampled every 4 msec.

Data are reported as the mean ± the standard error of the mean (SEM). For statistical comparisons, significance was defined at the 95% confidence level.

Results

Effect of pH on $InsP_3$-activated Channel Gating

To determine whether free hydroxide concentration could affect the gating of $InsP_3$-activated channels, pH modified solutions of 2.4×10^{-7} M $InsP_3$ were applied to the inside face of 6 cell-free patches of ORN membrane. Both channels were pH insensitive between pH 6 and pH 8. The open probability (Pr_{open}) of the 30 pS $InsP_3$-gated channel then increased between pH 8 and 11 from 0.05 ± 0.03 to 0.62 ± 0.08; a change of approximately 12 fold (n=4). The 74 pS $InsP_3$-gated channel showed a similar increased Pr_{open} between pH 8 and 11 from 0.07 ± 0.02 to 0.36 ± .14; a change of approximately 5 fold (n=2) (Figure 6-1).

Appearance of Modal Patterns Differing in Gating Kinetics

In approximately ten percent of all patches recorded (11 of 110), both the 30 (Figure 6-2) and 74 pS $InsP_3$-gated channel (data not shown) entered long duration open modes. Channels left their most observed state ($Pr_{open} \approx 0.33$) and entered one of three kinetically different modes. Modes were defined based on cutoff open probability as follows; Mode 1, Pr_{open} was > 0.90, Mode 2, the channel maintained a sustained open state ($Pr_{open} = 1.0$) for a second or longer, and Mode 3, Pr_{open} was ≤ 0.15.

The different patterns of gating can be attributed to the mean open and closed dwell-time in both the 30 and 74 pS $InsP_3$-gated channels (Figure 6-3). The sampling size of the mean open (t_o) and closed time (t_c) for channel modes varied because not all 11 patches contained all three modal patterns and because reliable estimates of t_o and t_c could not be made for modes having extremely low Pr_{open} or long open durations. This small sampling size, prevented statistical comparison of the mean open or closed duration across modes and between the two conductance channels.

Figure 6-1. Effect of pH on the gating of inositol 1,4,5-trisphosphate ($InsP_3$)- activated ion channels in lobster olfactory receptor neurons (ORNs).
(A) Unitary currents of the large-conductance (74 pS) $InsP_3$-gated channel at increasing pH values. Channel activity was recorded by applying 2.4×10^{-7} M $InsP_3$ to the inside face of cell-free patches of membrane voltage-clamped at -60 mV. Pipette and bath solution was symmetrical = control KA patch (see Table 1). Open probability (Pr_{open}), calculated from curve-fit amplitude histogram distributions of these records, is reported on the right. C= closed state, O= open state.
(B) Line graph showing mean ± SEM Pr_{open} for the small (30 pS)-(--▲--▲--)(n=4) and large (74 pS)-(--■--■--) (n=2) conductance $InsP_3$-gated channel as a function of pH.

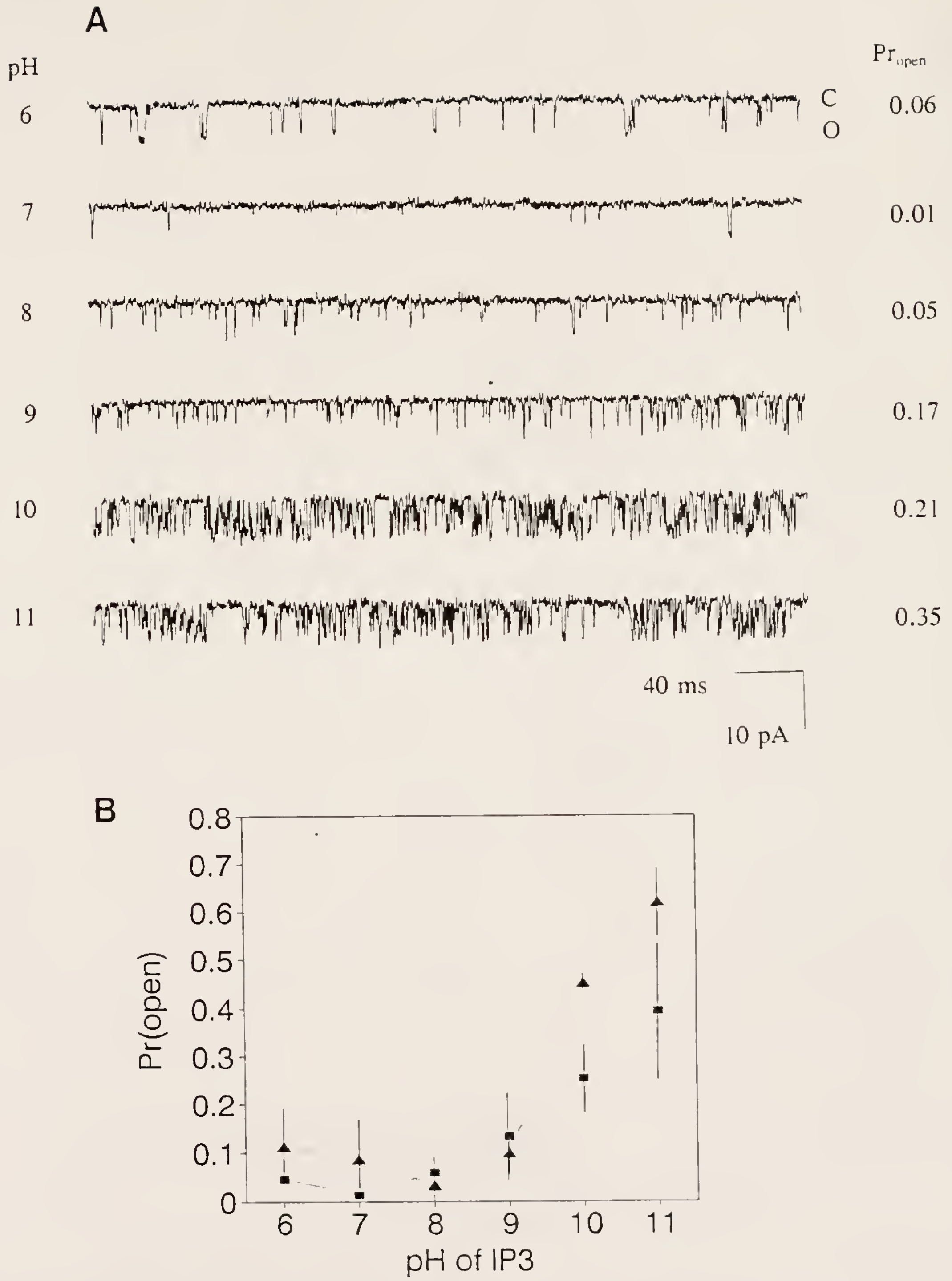
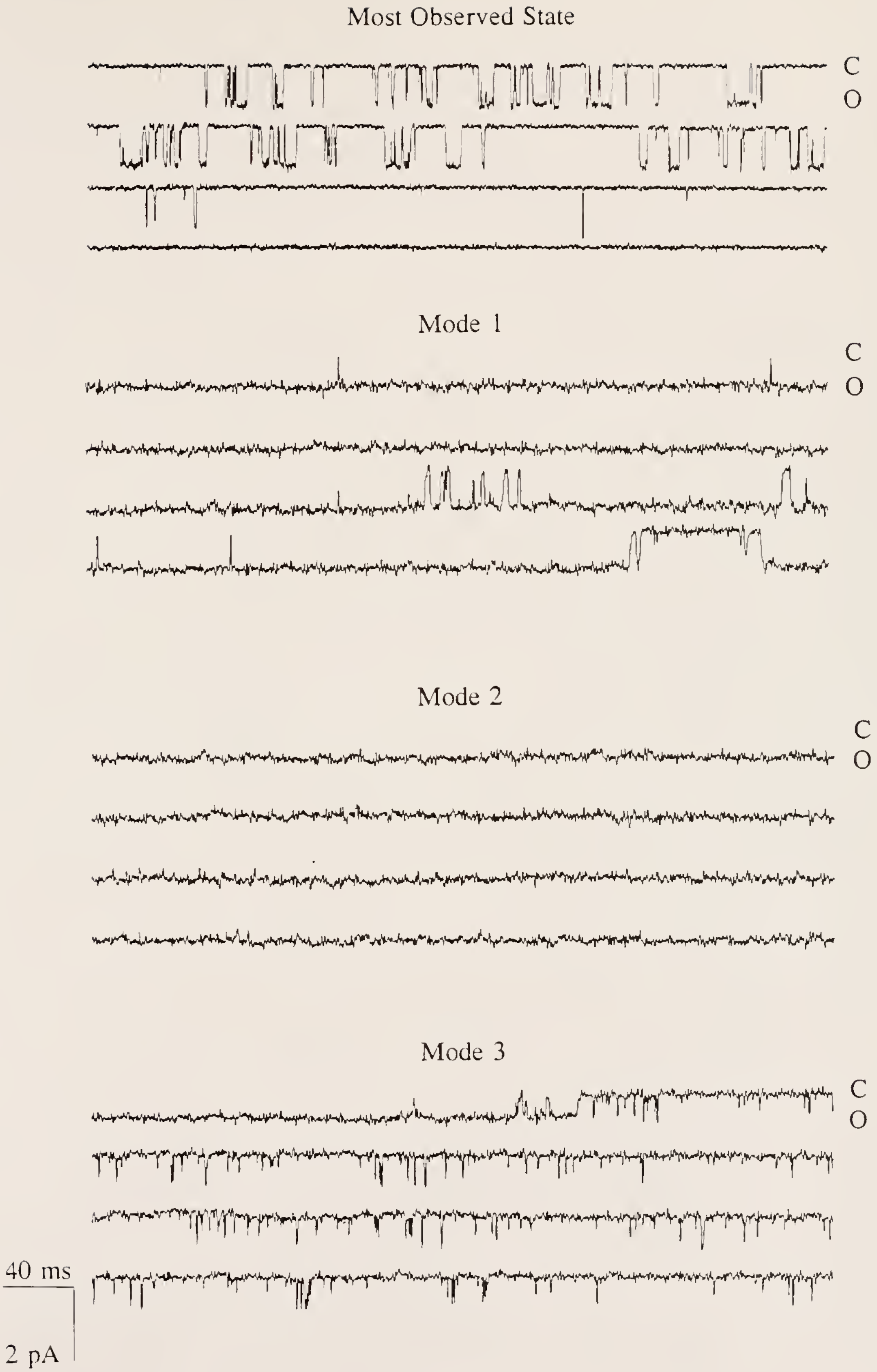

A
pH
Pr$_{open}$
6
C
O
0.06
7
0.01
8
0.05
9
0.17
10
0.21
11
0.35
40 ms
10 pA
B
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pr(open)
6
7
8
9
10
11
pH of IP3

Figure 6-2. Unitary currents of the small-conductance $InsP_3$-gated channel in lobster ORNs shown expressing three different kinetics: Mode 1 = long open duration, $Pr_{open} \geq 0.90$; Mode 2 = maintained open state, $Pr_{open} = 1.0$; Mode 3 = long closed state with rapid flicker to open state, $Pr_{open} = 0.15$. The most observed kinetic state (Mt Obs) is shown for comparison ($Pr_{open} \approx 0.33$). Similar mode behavior is observed in the large-conductance $InsP_3$-gated channel (data not shown). C, O, Pr_{open}, recording conditions and configuration as in Figure 6-1.



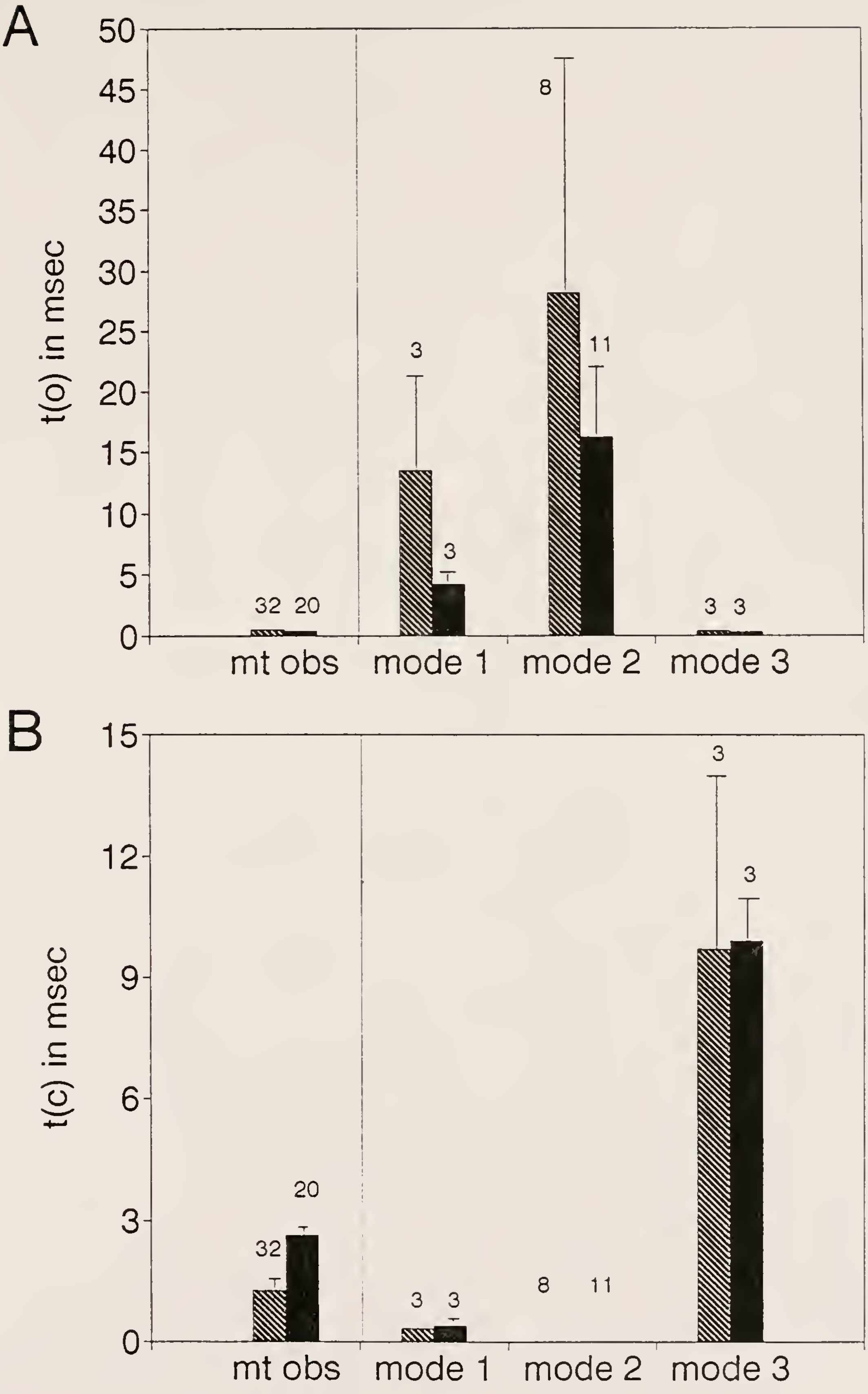
Most Observed State

Mode 1

Mode 2

Mode 3

Figure 6-3. Histogram of the mean open (t_o) and close (t_c) times of the small- (striped bar) and large- (solid bar) conductance $InsP_3$-gated channels as a function of mode. Data were calculated based on single exponential fits of the open and closed dwell-time histogram after separating into Mode 1 ($Pr_{open} > 0.90$), Mode 2 ($Pr_{open} = 1.0$, and Mode 3 ($Pr_{open} < 0.15$ with flickery transitions). The most observed state (mt obs; $Pr_{open} \approx 0.33$) is shown for comparison. Error bar ± SEM for sample size as noted. Note that values for Mode 2 were divided by 100 to fit to scale.


A
t(o) in msec
0
5
10
15
20
25
30
35
40
45
50
32
20
3
3
8
11
3
3
mt obs
mode 1
mode 2
mode 3
B
t(c) in msec
0
3
6
9
12
15
32
20
3
3
8
11
3
3
mt obs
mode 1
mode 2
mode 3

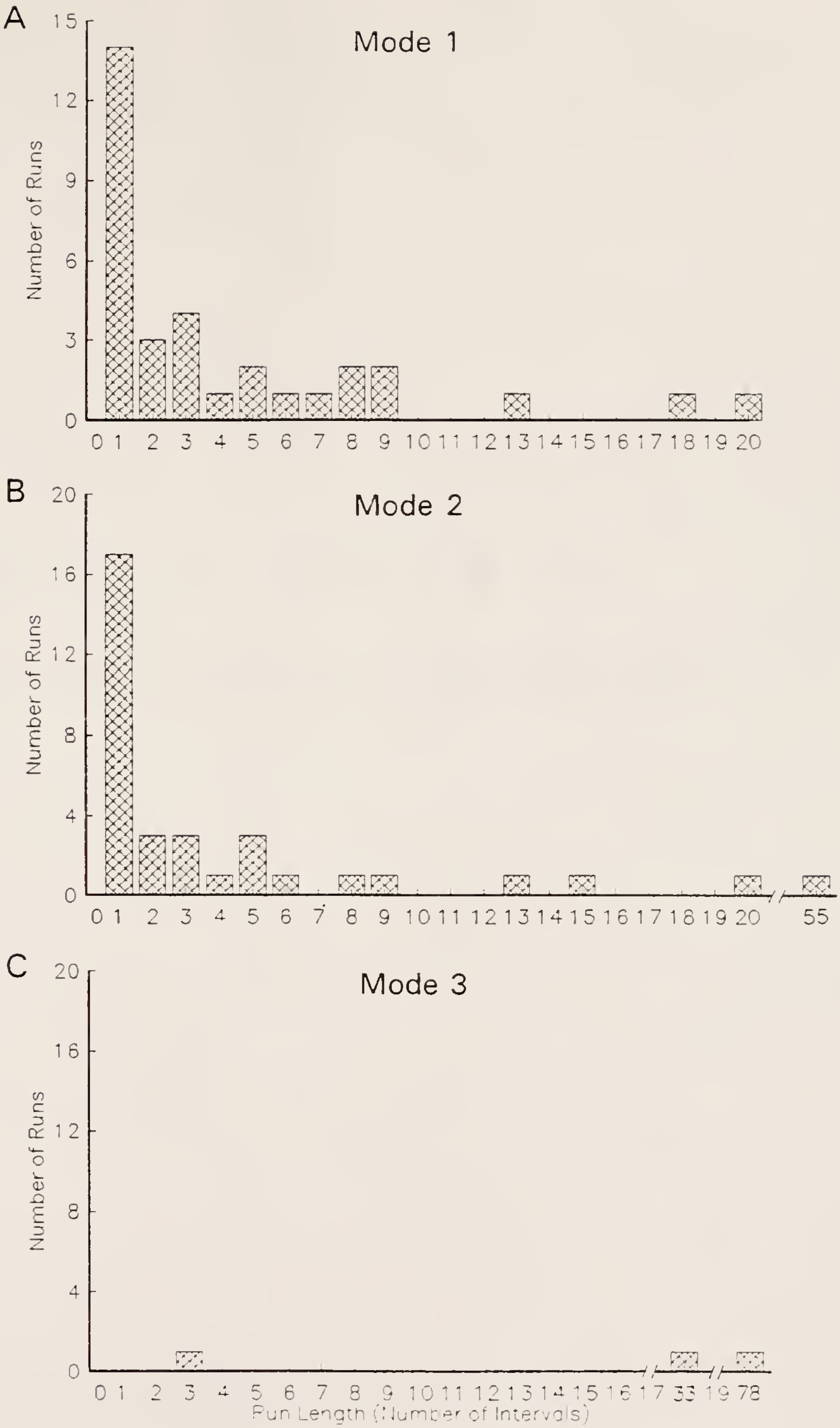
Qualitatively, exponential time constants governing the rapid transitions from open to closed state are strikingly different between the three modes and the most observed state but not between the two $InsP_3$-gated channels, independent of kinetic state (Figure 6-3). Modes 1 and 2 had a mean open time constant 10 and 1000X greater than that in the most observed state (Figure 6-3A) with mean closed time constants as much as 3X less than that in the most observed state (Figure 6-3B). The change in gating kinetics of Mode 3 could be solely attributed to a 3X increase in t_c over that in the most observed state, since t_o was unaffected. Unlike the most observed state, the kinetics of the modal patterns showed variability from one patch to the next; mean values for the 30 pS $InsP_3$-gated channel were: Mode 1 - $t_o = 129.06 \pm 59.18$ msec, $t_c = 0.31 \pm 0.03$ msec, Mode 2 - $t_o = 2814.75 \pm 1901.5$ msec, $t_c = 0$ msec, and Mode 3 - $t_o = 0.26 \pm 0.05$ msec, $t_c = 9.7 \pm 4.21$ msec. The mean values for the 74 pS $InsP_3$-gated channel were: Mode 1 - $t_o = 52.91 \pm 20.67$ msec, $t_c = 0.40 \pm 0.08$ msec, Mode 2 - $t_o = 1620.62 \pm 570.9$ msec, $t_c = 0$ msec, and Mode 3 - $t_o = 0.28 \pm 0.07$ msec, $t_c = 9.91 \pm 0.89$ msec.

Once channels progressed into one of the three defined modes (Figure 6-2), spontaneous transitions from one mode to another were infrequent, with sojourns ranging 1-21 seconds in each mode. One method to discern whether the modal gating was a random or ordered process would be to perform a runs analysis. Runs analysis of two 74 pS $InsP_3$-gated channels and one 30 pS $InsP_3$-gated channel demonstrated that the channels displayed modes of gating that were clustered; a given mode of gating was maintained for an arbitrarily defined time period before spontaneously changing to another mode (Figure 6-4). Transitions between modes, however, were random, i.e., independent of the previous history of gating, with the exception of Mode 3, which always exited to the most observed state. This is best exemplified in a contingency table of $InsP_3$ channel gating, in which the number of time intervals that a particular mode is followed by the same or different mode is tabulated (Figure 6-4). Analysis of the diagonal (double line box) demonstrated that the observed (italics) frequencies of repeats of the same type of gating mode was significantly different than the expected (bracketed) if the pattern of gating was purely random (Chi-square, $p \leq 0.005$). Highly diagonalized contingency tables were obtained for the other two $InsP_3$-gated channels analyzed in this manner (data not shown). Secondly, the observed frequency of entering or exiting Mode 3 from another state was always one or zero; significantly different than the expected (Chi-square, $p \leq 0.005$). This was consistent with the non-random behavior of the channel entering Mode 3 through Mode 2, and then finally exiting Mode 3 to either a blank or the most observed state. Frequency distributions were used to yield information about

Figure 6-4. Four x four contingency table of small-conductance $InsP_3$-channel mode gating. The gating pattern of interval (K + 1) is shown in relation to gating of interval K; interval length was arbitrarily set to 300 ms, N= 326 intervals classified. The observed numbers of intervals in each category are in italics; expected numbers for random occurrence in brackets. Expected numbers were calculated as the product of individual modal probabilities times the total number of intervals. Chi-square significance $p \leq 0.005$ (shaded boxes). Similar analysis yielded comparable results for large-conductance $InsP_3$-channel mode gating (table not shown).

	Blanks	Mode 1	Mode 2	Mode 3
Blanks	*0* [< 1]	*0* [< 1]	*0* [< 1]	*1* [1]
Mode 1	*0* [< 1]	*45* [18]	*16* [27]	*0* [31]
Mode 2	*0* [< 1]	*18* [27]	*131* [41]	*1* [47]
Mode 3	*1* [1]	*0* [31]	*0* [47]	*109* [55]

Figure 6-5. Distribution histograms of run lengths for mode gating in $InsP_3$ channels, constructed by pooling data from three patches (1 small, 2 large conductance). A run = A series of intervals displaying channel activity in the same mode. The duration of the sojourns in each mode was calculated by multiplying the mean number of intervals (exponential fit of the distribution) by 307.20 ms, the defined, arbitrary interval duration. Calculated time constants: Mode 1= 1.49 ± 0.09 s, Mode 2= 0.88 ± 0.03 s, and *Mode 3= 11.76 ± 6.7 s. * = Calculated average duration of three runs.


A
Mode 1
Number of Runs
0
3
6
9
12
15
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
Mode 2
Number of Runs
0
4
8
12
16
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 55
C
Mode 3
Number of Runs
0
4
8
12
16
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 33 19 78
Run Length (Number of Intervals)

the duration a channel remained in one modal pattern of gating before switching to another (Figure 6-5). Histograms of the length of a run of a single type of modal gating were well fit by a single exponential for Mode 1 and Mode 2. The run length for Mode 3 was not exponential, implying an exit rate that did not follow a random or Markov process.

Effect of Ca^{2+} on $InsP_3$-activated Channel Gating

Although neither $InsP_3$-activated channel could be gated directly by 1mM $[Ca^{2+}]_i$ or 13 mM $[Ca^{2+}]_o$, as evidenced by the lack of channel activity evoked in either Ca patch or PS solution, the Pr_{open} as well as the unitary conductance was dependent upon the Ca^{2+} concentration of the $InsP_3$ solution. The $InsP_3$-gated channels did not represent Ca^{2+}-activated channels, such as those mediating calcium-induced calcium release (CICR), but rather Ca^{2+} modulated, $InsP_3$-activated channels. When the inside face of the patch was challenged with 2.4×10^{-7} M $InsP_3$, three of four 30 pS channels (Figure 6-6A) and two of two 74 pS channels (Figure 6-6B) increased Pr_{open} between $[Ca^{2+}]_i$ of 0.001 and 1.0 mM. Further increasing the $[Ca^{2+}]_i$ from 1.0 mM to 30 mM reduced the slope conductance of each channel type (Figure 6-6). The reduction in slope conductance under high $[Ca^{2+}]_i$ was generally observed across the entire recorded voltage range (Figure 6-7A), but in two instances was due to a change from inward rectification, and therefore only varied at depolarizing potentials (Figure 6-7B).

Figure 6-6. Effect of Ca^{2+} on the gating of $InsP_3$-gated ion channels in lobster ORNs. Line graph of the open probability (Pr_{open}) (--▲--▲--) and the slope conductance (slope g)(--■--■--) of a small conductance (A) or a large conductance (B) $InsP_3$-gated channel as a function of Ca^{2+} concentration. Recording conditions and configuration as in Figure 6-1.

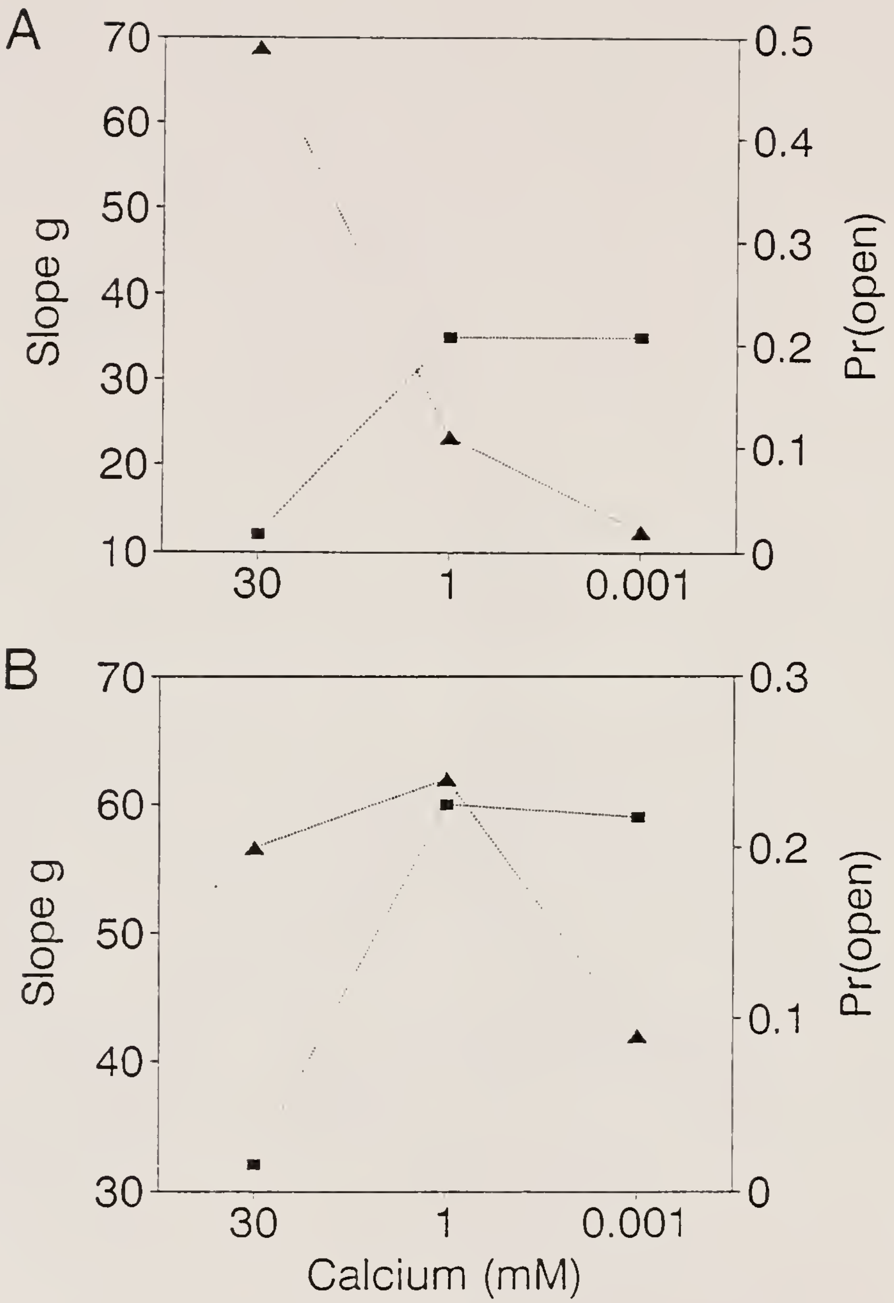
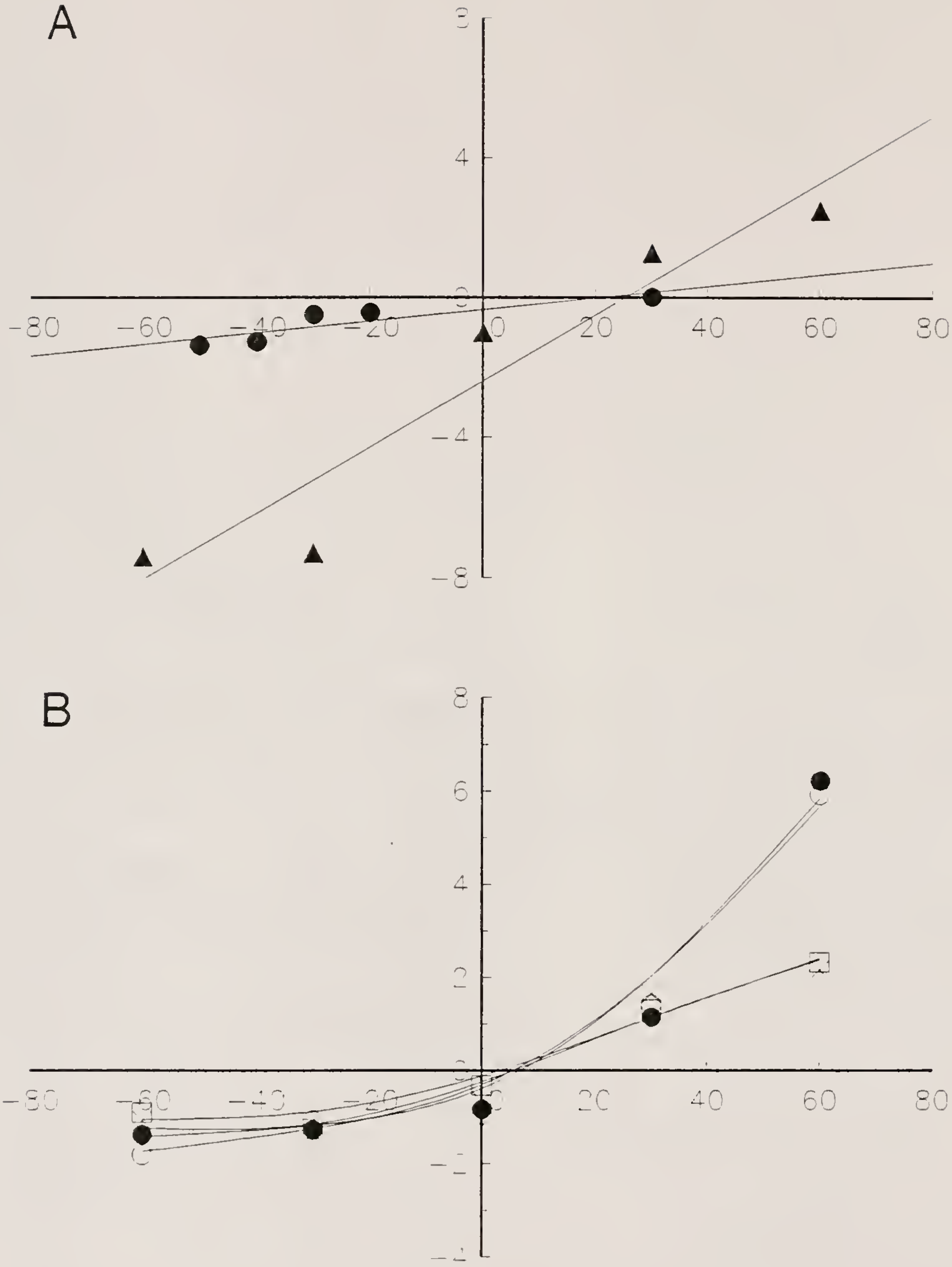

A
70
60
50
40
30
20
10
Slope g
0.5
0.4
0.3
0.2
0.1
0
Pr(open)
30
1
0.001
B
70
60
50
40
30
Slope g
0.3
0.2
0.1
0
Pr(open)
30
1
0.001
Calcium (mM)

Figure 6-7. Effect of Ca^{2+} on the conductance of $InsP_3$-gated ion channels in lobster ORNs.

(A) Current (ordinate)-voltage (abscissa) relation of a large conductance $InsP_3$-gated channel recorded by applying 2.4×10^{-7} M $InsP_3$ in 1.0 mM Ca^{2+} (—▲—▲—) or 30 mM Ca^{2+} (—●—●—) patch (see Table 6-1) to the inside face of cell-free patches of membrane.

(B) Current (ordinate)-voltage (abscissa) relation of a small conductance $InsP_3$-gated channel recorded by applying 2.4×10^{-7} M $InsP_3$ in 0.001 mM Ca^{2+} (—●—●—), 0.01 mM Ca^{2+} (—o—o—), 0.1 mM Ca^{2+} (—□—□—), or 30 mM Ca^{2+} (—x—x—) patch (see Table 6-1) to the inside face of cell-free patches of membrane.

(A & B) Pipette solution = control KA patch (see Table 6-1); Patches were voltage-clamped at -60 mV.


A
8
4
0
-4
-8
-80
-60
-40
-20
0
20
40
60
80
B
8
6
4
2
0
-2
-4
-80
-60
-40
-20
0
20
40
60
80

In a limited number of trials, it was possible to apply $InsP_3$ at five different $[Ca^{2+}]$ to the inside face of three 30 pS and one 74 pS $InsP_3$-gated channel. Due to the difficulty of patch integrity through all 5 solution changes, the data from the 4 channels were pooled for the analysis of the effect of Ca^{2+} on channel Pr_{open}. Chelation with 11 mM EGTA reduced the Pr_{open} of the channels by 51 ± 13 percent (n=4) from that measured at 0.1 mM $[Ca^{2+}]_i$. Changing the $[Ca^{2+}]_i$ to 30 mM, increased the Pr_{open} to 157 ± 7 percent (n=4) of that found in 0.1 mM $[Ca^{2+}]_i$. Pr_{open} remained near that recorded in the 0.1 mM $[Ca^{2+}]_i$ condition when 30 mM Ba^{2+} was substituted for Ca^{2+} (n=4)(Figure 6-8).

Pharmacology of Macroscopic Odor-evoked Current & $InsP_3$-gated Channels

To test the alternative hypothesis that $InsP_3$ could mobilize intracellular Ca^{2+}, cells were preincubated with a membrane permeant, Ca^{2+}-ATPase pump inhibitor, thapsiagargin. The magnitude of inward odor-evoked macroscopic currents was not significantly different among cells preincubated with 8×10^{-7} M thapsiagargin and those preincubated with the EtOH carrier alone (Figure 6-9, Student's t-test).

Macroscopic inward odor-evoked currents that are mediated by $InsP_3$ (Chapter 5) were reversibly blocked by a mixture of two transition metals that block voltage-activated Ca^{2+} channels (5 mM Co/Cd). The inward currents were also partially and reversibly blocked by RR, a blocker

Figure 6-8. Histogram of the mean ± SEM open probability (Pr_{open}) of both the small- and large-conductance $InsP_3$-gated channel (n=4) in lobster ORNs as a function of internal Ca^{2+} concentration (2.4×10^{-7} M $InsP_3$ in Ca Patches). Configuration as in Figure 6-1. +EGTA = 0.001 mM Ca Patch + 11 mM EGTA; Ba30 = 30 mM Ba Patch. Pipette solution = control KA patch. Patches were voltage-clamped at -60 mV. See Table 6-1 for patch solution composition.

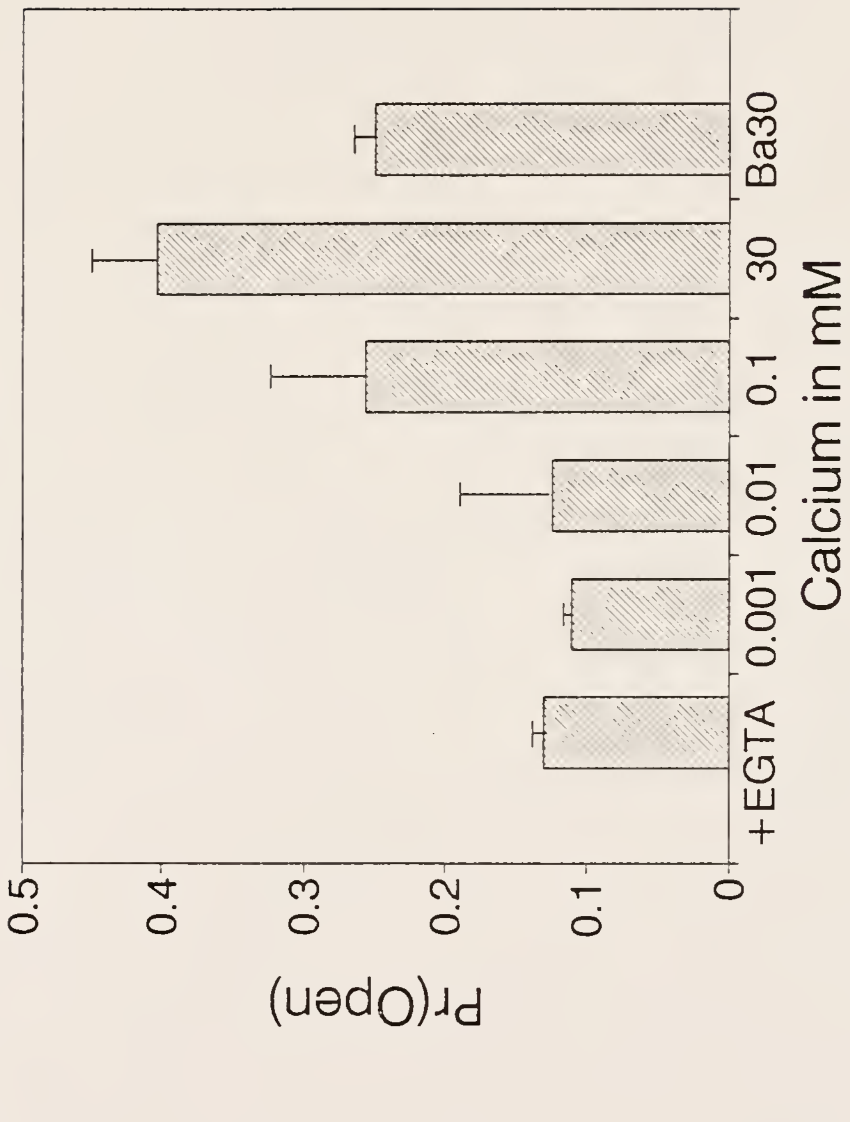
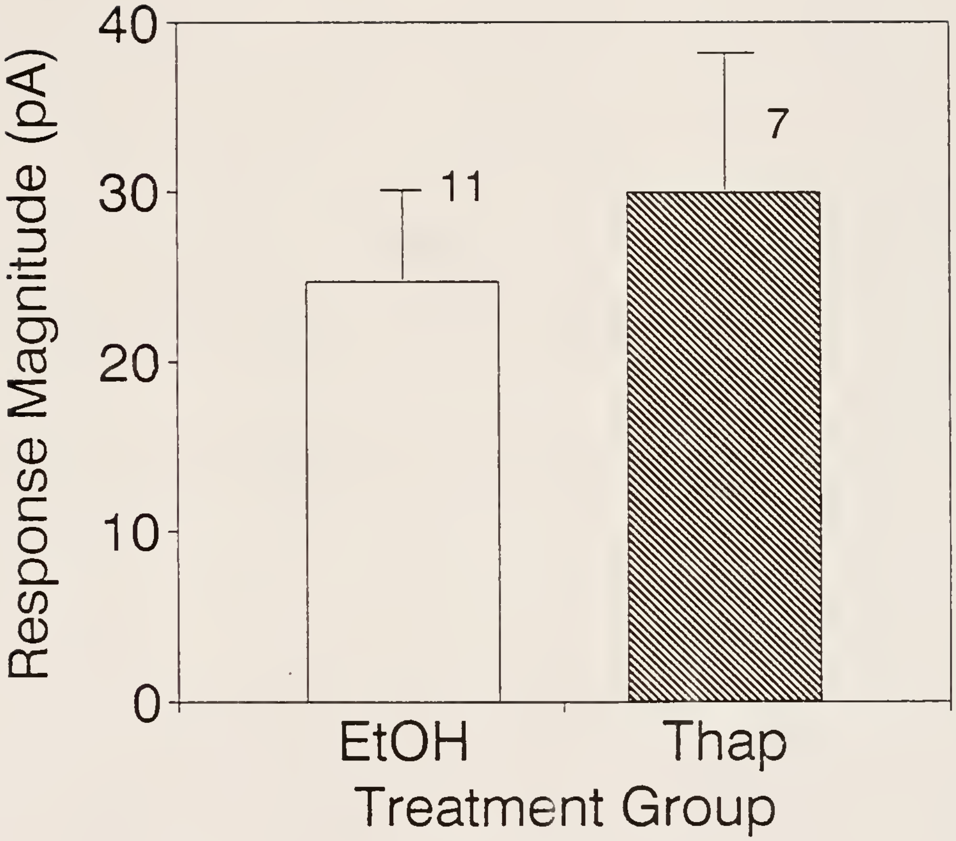

Pr(Open)
0.5
0.4
0.3
0.2
0.1
0
+EGTA
0.001
0.01
0.1
30
Ba30
Calcium in mM

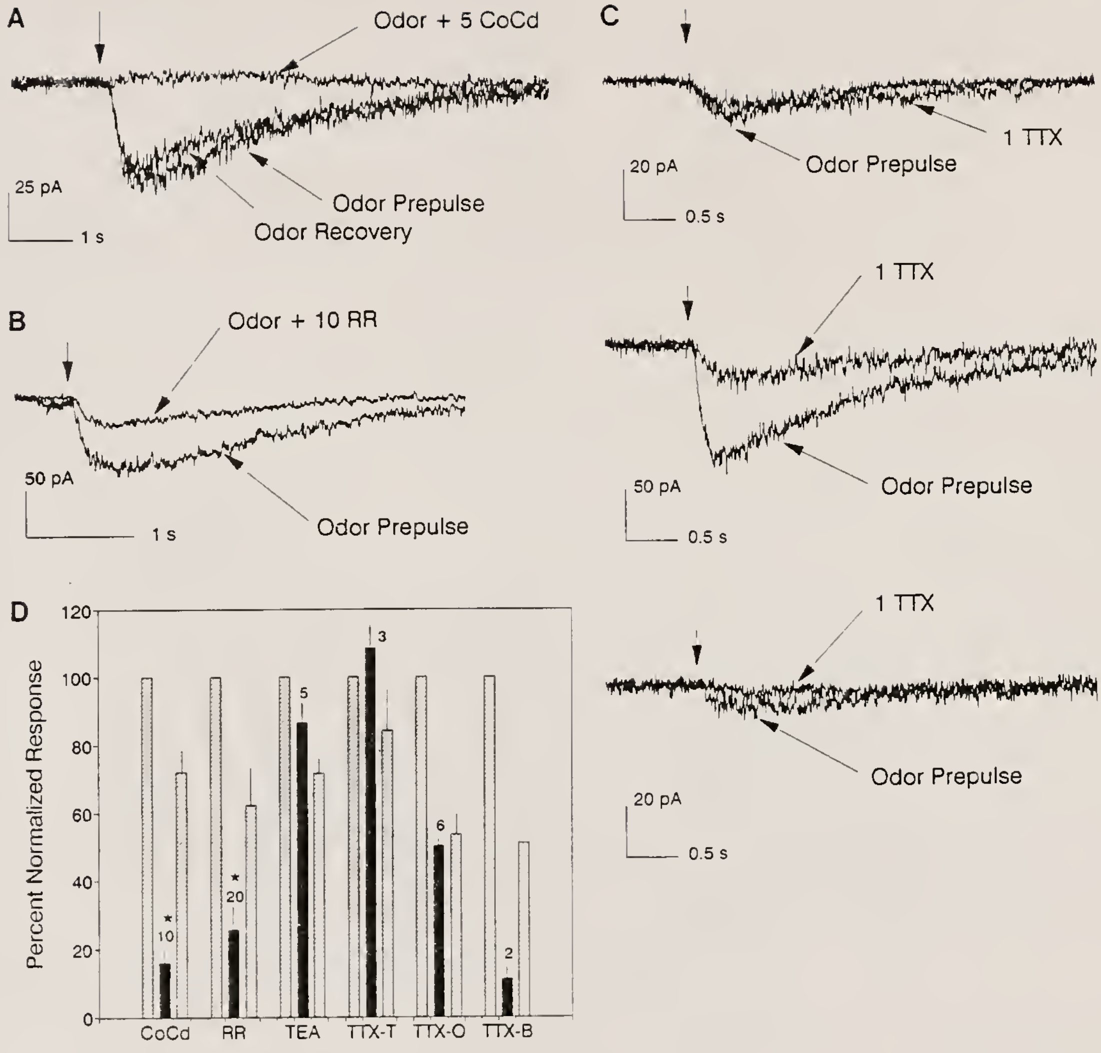
Figure 6-9. Histogram of the mean ± SEM macroscopic inward odor-evoked current in lobster ORNs treated with 8 x 10^{-7} M thapsiagargin (Thap) or with the EtOH carrier alone (EtOH) two hours prior to recording. ORNs were voltage-clamped at -60 mV. Odorants used to evoke the current include 10^{-3} M proline or a 1000X dilution of TET stock.


Response Magnitude (pA)
40
30
20
10
0
11
7
EtOH
Thap
Treatment Group

of $InsP_3$-regulated Ca^{2+} channels (10 µM RR). Co/Cd significantly reduced the peak current 84 ± 7.5 percent (n=10) while RR reduced it 74 ± 10.7 percent (n=20) of initial control values (arcsin transformation of percentage data followed by Student's t-test)(Figure 6-10A,B,D). Application of 5 mM TEA, a blocker of all known K^+ channels, reduced the odor-evoked inward current by only 13.5 ± 9 percent, which was not significant nor contributable to all cells tested (n=5)(Figure 6-10D). One micromolar TTX, an exceptionally specific blocker of voltage-activated Na^+ channels, had a heterogenous effect across 10 cells tested. TTX delayed the termination of the odor-evoked inward current (n=3), slowed the onset kinetics and reduced the magnitude of the current (n=6), or completely blocked the current (n=2)(Figure 6-10C,D).

The pharmacology of the $InsP_3$-activated unitary currents was consistent with that of the macroscopic odor-evoked inward current, further supporting that $InsP_3$-gated channels underlie the excitatory current. Five millimolar Co\Cd blocked 3 of 4 tested 30 pS channels and 3 of 4 tested 74 pS channels. Ten micromolar RR consistently blocked both the 30 pS channel (n=3) and the 74 pS channel (n=2). One micromolar TTX blocked 2 of 4 tested 30 pS channels and did not block the 74 pS channel (n=4). TEA was not tested for its effect on unitary currents. Ten millimolar Cs was not tested for its effect on macroscopic currents but failed to

Figure 6-10. Pharmacological probes targeting the macroscopic inward odor-evoked current in lobster ORNs.
(A) Whole-cell current evoked by application (arrow) of the odorant TET (Odor Prepulse), TET plus 5 mM Co and 5 mM Cd (Odor + 5 CoCd), and then the odorant TET (Odor Recovery).
(B) Whole-cell current evoked by application (arrow) of the odorant TET (Odor Prepulse), TET plus 10 μM ruthenium red (Odor + 10 RR).
(C) Whole-cell current evoked by application (arrow) of the odorant TET (Odor Prepulse), TET plus 1 μM tetrodotoxin (Odor + 1 TTX). Top trace: TTX appears to affect termination of the odor response (TTX-T). Middle trace: TTX appears to affect the onset and magnitude of the odor response (TTX-O). Bottom trace: TTX appears to affect the termination of the odor response (TTX-T).
(D) Histogram plot of the mean ± SEM macroscopic inward odor-evoked current of ORNs treated with various pharmacological probes (solid bars). Odor Prepulse = striped bar preceding each treatment, Odor Recovery = striped bar following each treatment. TEA = 5 mM tetraethylammonium. * = Significant difference, paired t-test. Statistics not computed for heterogeneous effect of TTX. TTX-T, TTX-O, TTX-B as in part C.
(A-D) ORNs voltage-clamped at a -60 mV holding potential. Odorants used to evoke the current include 10^{-3} M proline, taurine, glycine, betaine, or a 1000X dilution of TET stock.


A
Odor + 5 CoCd
Odor Prepulse
Odor Recovery
25 pA
1 s
B
Odor + 10 RR
Odor Prepulse
50 pA
1 s
C
1 TTX
Odor Prepulse
20 pA
0.5 s
1 TTX
Odor Prepulse
50 pA
0.5 s
1 TTX
Odor Prepulse
20 pA
0.5 s
D
Percent Normalized Response
120
100
80
60
40
20
0
CoCd
RR
TEA
TTX-T
TTX-O
TTX-B
★
10
★
20
5
3
6
2

block either the 30 pS channel (n=5) nor the 74 pS channel (n=3).

Ionic Selectivity of $InsP_3$-gated Channels

The mean reversal potential for the odor-evoked inward current was -46.5 ± 2.1 mV (n=26), over 80 mV negative of the calculated Nernst potential for Ca^{2+} (E_{Ca} = 34 mV) (Figure 6-11), suggesting that the excitatory odor-evoked inward current is a mixed current. The reversal potential of twenty-one 30 pS $InsP_3$-gated channels and six 74 pS $InsP_3$-gated channels were evaluated using two different ion substitution paradigms. In the first paradigm, the calculated Nernst potential for Cl^-, Na^+, or K^+ was shifted varying degrees from 0 mV and the Nernst potential for Ca^{2+} was set to 0 mV. Eleven of 15 30 pS channels and 3 of 4 74 pS channels reversed near 0 mV, under these conditions. An example of one of the 30 pS channels is given in Figure 6-12. In symmetrical conditions the channel reversed at 0.11 mV (not shown). When E_{Ca} = 0, E_{Na} = -19, and E_{Cl} = 30, the channel reversed at 2.9 mV. When E_{Ca} = 0, E_{Na} = -26, and E_{Cl} = 28, the channel reversed at 4.9 mV. An example of one of 74 pS channels is given in Figure 6-13. In symmetrical conditions the channel reversed at 4.5 mV (not shown). When E_{Ca} = 0, E_{Na} = -19, and E_{Cl} = 30, the channel reversed at 8.8 mV. When E_{Ca} = 0, E_{Na} = -26, and E_{Cl} = 28, the channel reversed at 10.7 mV. One of the three 74 pS channels displayed extreme inward rectification at positive

Figure 6-11. Current reversal of macroscopic odor-evoked inward currents in lobster ORNs.
(A) Odorant-evoked (TET) whole-cell currents in a lobster ORN voltage-clamped at various membrane potentials.
(B) Histogram plot of the current reversal for 26 such ORN recordings binned into 10 mV holding potential intervals (V_c Range). Mean reversal potential = -46.5 ± 2.1 mV. Odorants applied to evoke the current include (10^{-3} M) AMP, glycine, proline, taurine, or betaine, or a 1000X dilution of TET stock.

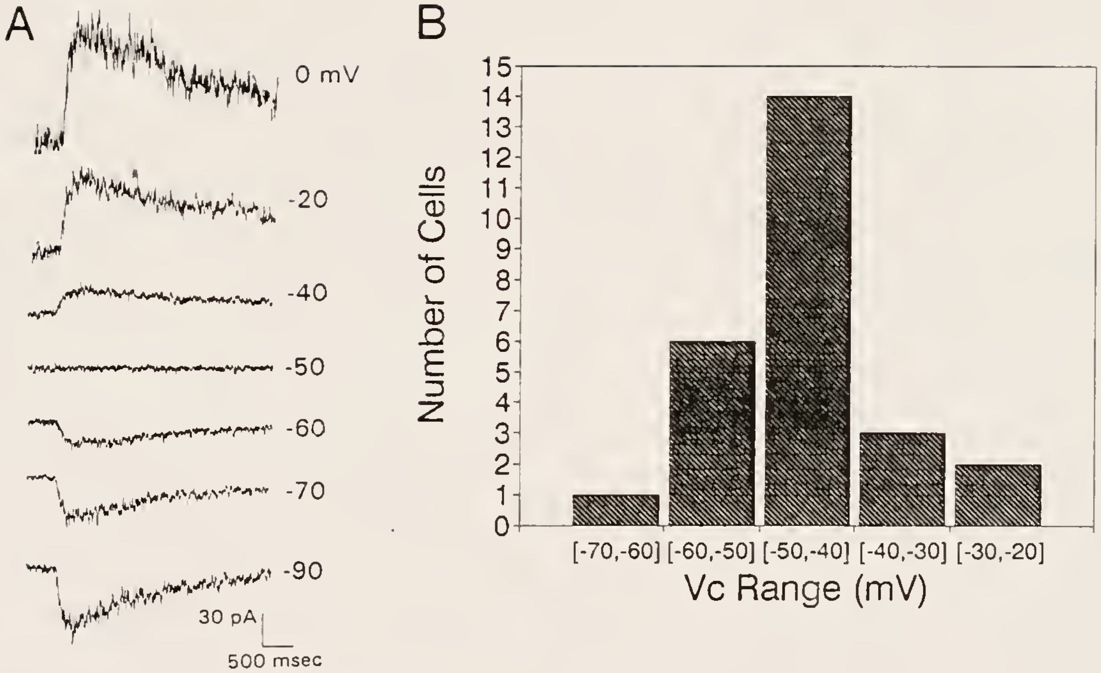

A
0 mV
-20
-40
-50
-60
-70
-90
30 pA
500 msec
B
Number of Cells
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
[-70,-60]
[-60,-50]
[-50,-40]
[-40,-30]
[-30,-20]
Vc Range (mV)

Figure 6-12. Current reversal of the small- (30 pS) conductance InsP$_3$-gated channel in a lobster ORN recorded in ionically substituted solutions.

(A) Unitary currents evoked by 2.4 x 10^{-7} M InsP$_3$ in Na100Cs Bath (—□—□—) or Na Bath (—●—●—) when applied to the inside-face of a cell-free patch of lobster ORN voltage-clamped at various membrane potentials. C= closed state, O$_1$= first open state, O$_2$= second open state, O$_3$= third open state. See Table 6-1 for bath solution composition.

(B) Current (ordinate)-voltage (abscissa) relation of the small-conductance InsP$_3$-gated channel in A. Each data point represents at least 12s of open channel events binned into a point-by-point amplitude histogram, which was fitted by a gaussian curve. The height of the fitted distribution determined the mean unitary current (in pA) and was plotted as a function of voltage (in mV). The plotted relation was fitted by linear regression (continuous line). Calculated Nernst potentials for various ion species are denoted by the arrows and specified in text. E$_{Ca}$ was set to zero unless noted otherwise. Symbols as in A.

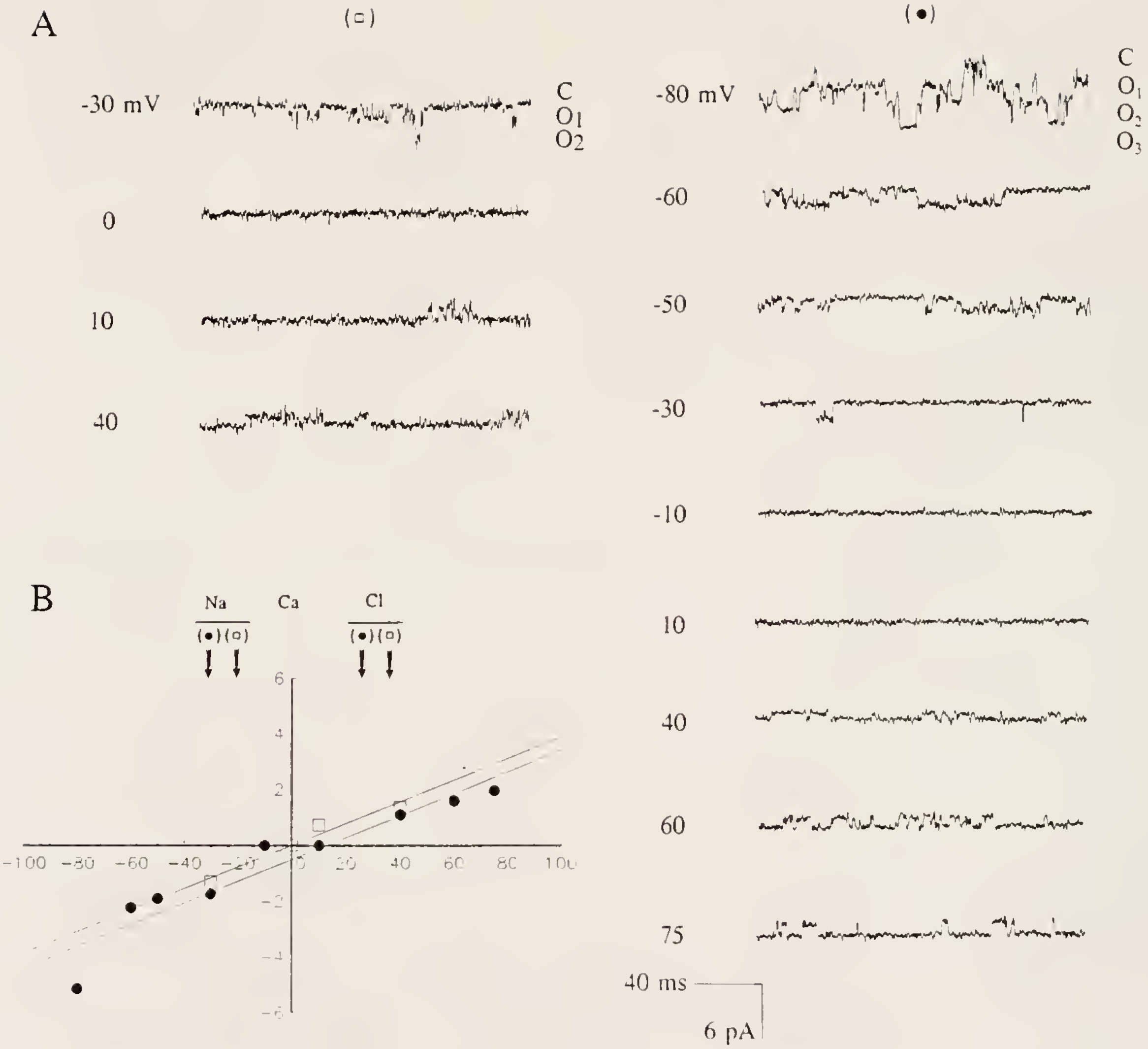

A
(□)
(●)
-30 mV
0
10
40
C
O1
O2
-80 mV
-60
-50
-30
-10
10
40
60
75
C
O1
O2
O3
40 ms
6 pA
B
Na
(●) (□)
Ca
Cl
(●) (□)
6
4
2
-2
-4
-6
-100
-80
-60
-40
-20
0
20
40
60
80
100

Figure 6-13. Current reversal of the large- (74 pS) conductance $InsP_3$-gated channel in a lobster ORN recorded in ionically substituted solutions.
(A) Unitary currents evoked by 2.4 x 10^{-7} M $InsP_3$ in Na Bath (—●—●—) or Na100Cs Bath (—o—o—) when applied to the inside-face of a cell-free patch of lobster ORN voltage-clamped at various membrane potentials.
(B) Current (ordinate)-voltage (abscissa) relation of the large-conductance $InsP_3$-gated channel in A.
(A & B) Analysis and notation as in Figure 6-12.

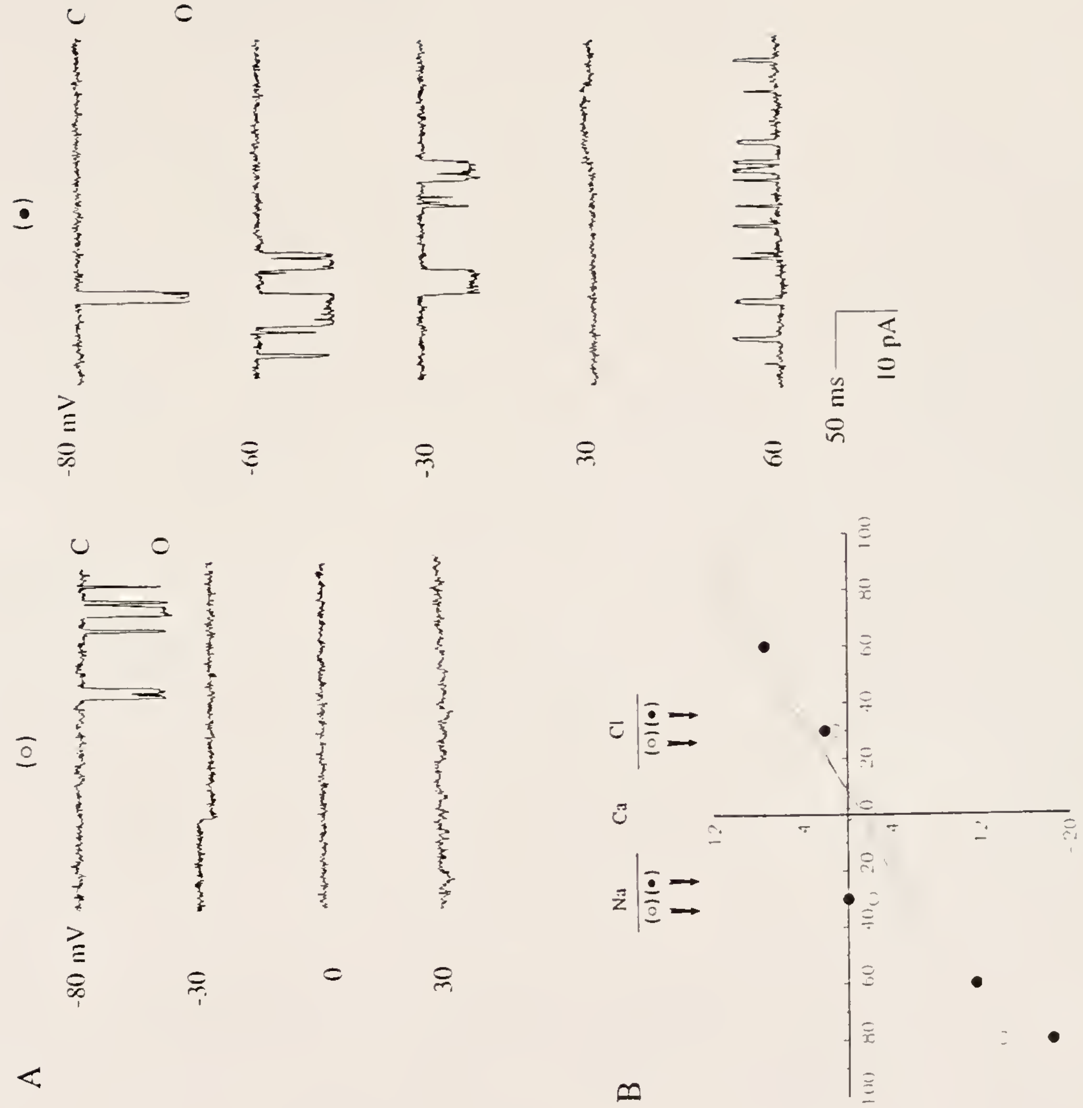
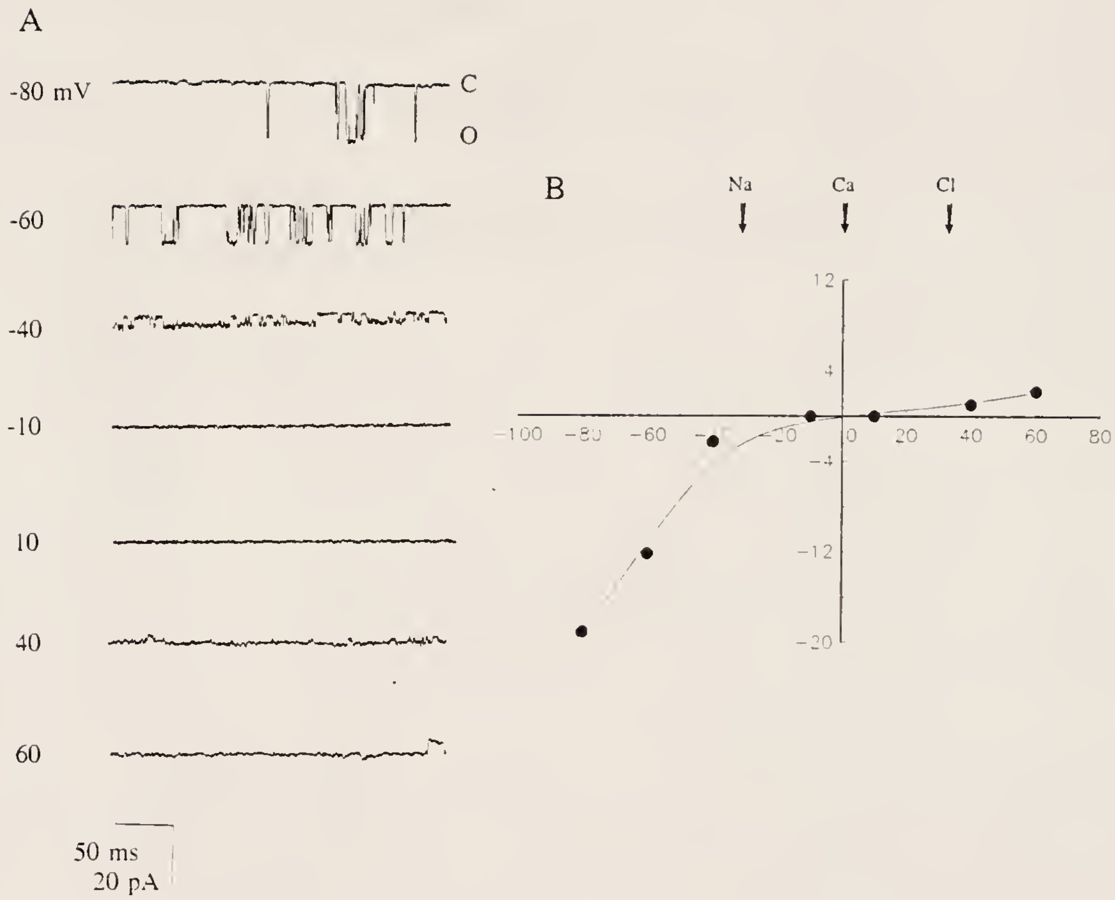

A
(○)
-80 mV
-30
0
30
C
O
(●)
-80 mV
-60
-30
30
60
C
O
50 ms
10 pA
B
Na
(○)(●)
Ca
Cl
(○)(●)
100
80
60
40
20
0
20
40
60
80
100
12
4
4
12
20

Figure 6-14. Current reversal of the large- (74 pS) conductance InsP_3-gated channel in a lobster ORN recorded in ionically substituted solutions.

(A) Unitary currents evoked by 2.4×10^{-7} M InsP_3 in Ca Bath (—●—●—) when applied to the inside-face of a cell-free patch of lobster ORN voltage-clamped at various membrane potentials.

(B) Current (ordinate)-voltage (abscissa) relation of the large-conductance InsP_3-gated channel in A.

(A & B) Analysis and notation as in Figure 6-12.

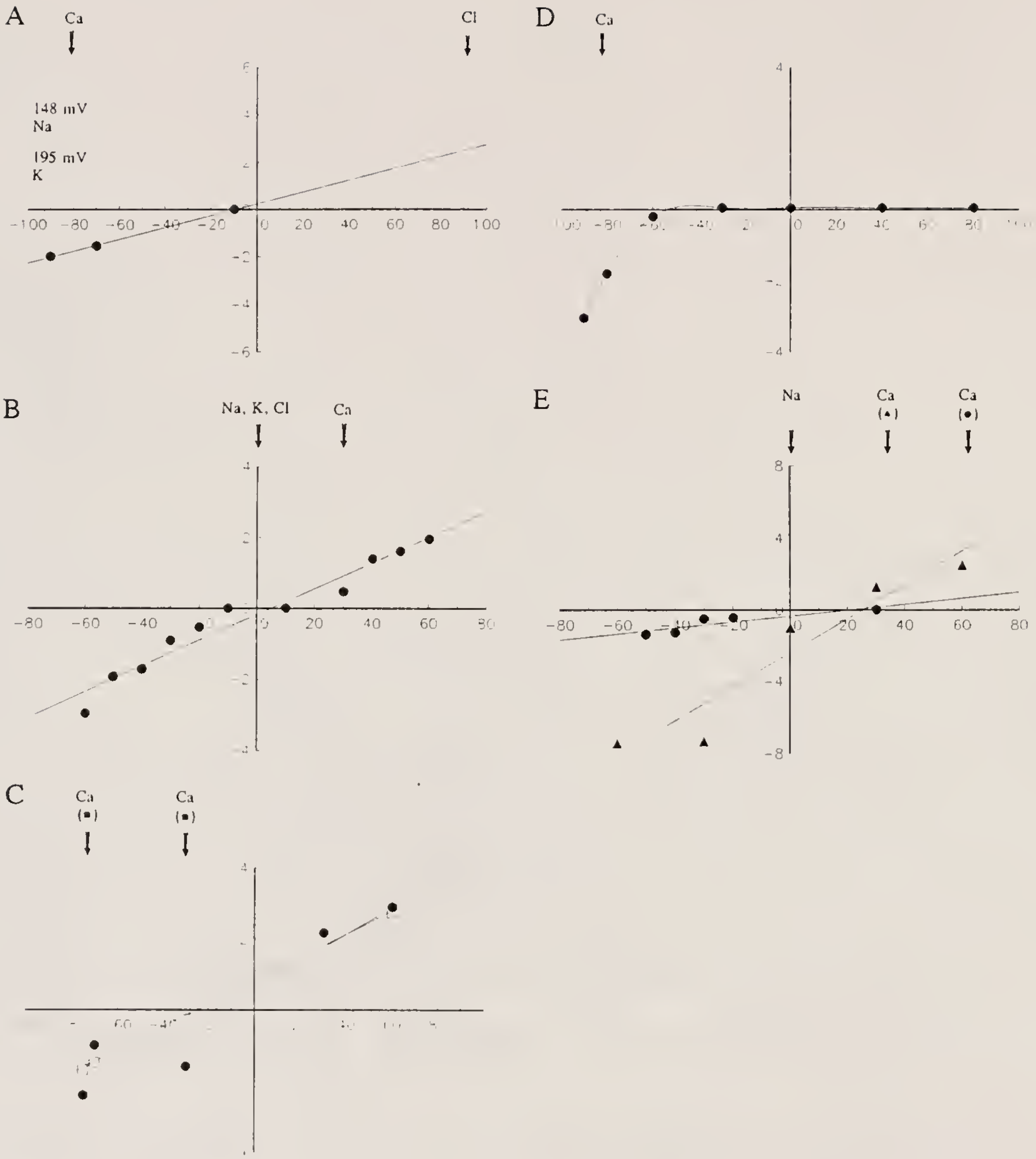

A
-80 mV
C
O
-60
-40
-10
10
40
60
50 ms
20 pA
B
Na
Ca
Cl
12
4
-4
-12
-20
-100
-80
-60
-40
-20
0
20
40
60
80

holding potentials (Figure 6-14). In symmetrical conditions, the channel reversed at 3.2 mV (not shown). When E_{Ca} = 0, E_{Na} = -26, and E_{Cl} = 25, the channel reversed at 4.6 mV.

The remaining 4 30 pS channels and one 74 pS channel that did not reverse near 0 mV, reversed independently of any calculated Nernst potential and were inconclusive as to selectivity (data not shown). For example, when E_{Ca} = 0 mV, E_{Cl} = 38.8 mV, E_{Na} = 148.6 mV, and E_K = 6.5 mV, the first of these remaining 30 pS channels reversed at 23 mV. In the second 30 pS channel, when E_{Ca} = 0 mV, E_{Cl} = 45.3 mV, E_{Na} = 148.6 mV, and E_K = 6.5 mV, the channel reversed at -38 mV. In the third remaining 30 pS channel, when E_{Ca} = 0 mV, E_{Na} = -72.3 mV, E_{Cl} = 70.6 mV, and E_K = 195.3 mV, the channel reversed at -15.7 mV. In the fourth remaining 30 pS channel, when E_{Ca} = 0 mV, E_{Na} = -23 mV, E_{Cl} = 25 mV, and E_K = -195.3, the channel reversed at -14.5 mV. In the remaining 74 pS channel, when E_{Ca} = 0 mV, E_{Na} = 46.7 mV, E_{Cl} = -28 mV, and E_K = -195 mV the channel reversed at -43 mV.

In the second substitution paradigm, E_{Ca} was shifted from 0 mV in order to discriminate between channels with primarily Ca^{2+} permeability and non-selective cation permeability. In conditions where E_{Ca} was other than 0 mV, channels of both conductances either followed E_{Ca}, remained at 0 mV, or were intermediate between E_{Na} and E_{Ca}

Figure 6-15. Current (ordinate)-voltage (abscissa) relation of the small- (A-C) and large- (D-E) conductance InsP$_3$-gated channel when 2.4×10^{-7} M InsP$_3$ was applied to the inside-face of cell-free patches of lobster ORN under the following ionic substitutions to force calculated E_{Ca} to be other than 0 mV. All other ions (E_x) at 0 mV unless shown otherwise.
(A) InsP$_3$ in Ca Bath (—●—●—)
(B) InsP$_3$ in Ca Patch (—●—●—) 0.01 mM
(C) InsP$_3$ in Ca Patch (—●—●—) 1.0 mM, (—□—□—) 30 mM
(D) InsP$_3$ in Ca Bath (—●—●—)
(E) InsP$_3$ in Ca Patch (—▲—▲—) 1.0 mM, (—●—●—) 30 mM
Analysis and notation as in Figure 6-12.


A
Ca
Cl
148 mV
Na
195 mV
K
-100
-80
-60
-40
-20
0
20
40
60
80
100
6
4
2
-2
-4
-6
D
Ca
4
-4
-80
-60
-40
0
20
40
80
B
Na, K, Cl
Ca
4
2
-2
-80
-60
-40
0
20
40
60
80
E
Na
Ca
(▲)
Ca
(●)
8
4
0
-4
-8
-80
-60
-40
-20
0
20
40
60
80
C
Ca
(■)
Ca
(■)
4
-60
-40

(Figure 6-15A-E). Three of six 30 pS channels reversed near 0 mV, implying non-selectivity. An example is given in Figure 6-15A. In symmetrical conditions the channel reversed at 10.1 mV (not shown). When E_{Ca} = -83, E_{Cl} = 88.6, E_{Na} = 148, and E_K = 195, the channel reversed at -9.6 mV. When E_{Ca} = 30 mV and the remaining ions were set to 0 mV, another 30 pS channel reversed at 3.4 mV (Figure 6-15B). The remaining three 30 pS channels reversed intermediate between E_{Na} and E_{Ca} such as the example in Figure 6-15C. In symmetrical conditions the channel reversed at 8.4 mV (not shown). When E_{Ca} = -30, E_{Cl}, E_{Na}, and E_K = 0, the current reversed at -19.5 mV. When E_{Ca} was further shifted to -74 mV, the current reversed at -28.7 mV.

Of the two 74 pS channels tested under conditions where E_{Ca} was other than 0 mV, one channel reversed consistent with E_{Ca} (Figure 6-15D) and the other channel reversed intermediate between E_{Na} and E_{Ca} (Figure 6-15E). In the example of the former, where E_{Ca} = -83, E_{Cl}, E_{Na}, and E_K = 0, the channel reversed at -55.8 mV with extreme rectification. In the latter example, in symmetrical conditions the channel reversed at 2.4 mV (not shown). When E_{Na} = 0 and E_{Ca} = 30, the channel reversed at 25 mV. However, when E_{Na} = 0 and E_{Ca} = 60, the channel reversed at 20.3 mV, intermediate between E_{Ca} and E_{Na}.

Discussion

Gating Properties

While pH sensitivity has not been investigated for cerebellar $InsP_3$-gated channels, pH modulates the kinetics of other ion channels, such as the I_{Ca} conductance of L-type Ca^{2+} channels (Prod'hom, Pietrobon, and Hess, 1987, 1989). Alkaline pH is known to increase binding affinity of the $InsP_3$ ligand to its receptor in isolated catfish olfactory ciliary membranes (Kalinoski et al., 1992). Under alkaline conditions, the 5 to 12 fold increase in Pr_{open} for the small- and large-conductance $InsP_3$-gated channel in lobster is not unlike the 6 fold increases in $InsP_3$ binding affinity observed in catfish olfactory cilia (Kalinoski et al., 1992). Therefore, physiological changes in pH could modify the gating of the $InsP_3$-activated channels in lobster ORNs by altering the affinity of $InsP_3$ binding.

The ability of Ca^{2+} to modulate both the small- and large-conductance $InsP_3$-gated channel in a concentration dependent manner is similar to the $InsP_3$-triggered Ca^{2+} flux in the cerebellum, which is stimulated at low $[Ca^{2+}]$ and inhibited at higher $[Ca^{2+}]$ (Ferris, Huganir, and Snyder, 1990; Iino and Endo, 1992; Mignery, Johnson, and Südhof, 1992; Luttrell, 1993). Yet oddly for $InsP_3$-gated channels in lobster ORNs, an increase in Pr_{open} of the channels in response to increased $[Ca^{2+}]_i$ is coupled with a decrease in channel conductance. These data suggest that Ca^{2+} could increase channel open frequency throughout the tested concentration range and concurrently, but independently, affect channel permeation at high Ca^{2+}, possibly by targeting the pore of the protein.

Cerebellar $InsP_3$ receptors are dependent on the Ca^{2+} content of the endoplasmic reticulum (Worley, Baraban, Colvin, and Snyder, 1987) and it has been suggested that calsequestrin, calmedin, or calreticulin may serve as the Ca^{2+} sensing unit, independent of the channel protein (Ferris et al., 1990; Berridge, 1993). Although the latter is conceivable for olfactory $InsP_3$ receptors, the former is irrelevant for lobster $InsP_3$-gated channels *in situ* (Ache, Hatt, Breer, Zufall, in press), which are located on outer dendritic processes of the ORN; an area devoid of organelles (Grünert and Ache, 1988). The fact that cultured lobster ORNs incubated with thapsiagargin demonstrated no change in odor-evoked inward currents, shown previously to be mediated by $InsP_3$ (Fadool and Ache, 1992a) and shown in this study to be carried partially by Ca^{2+}, suggests that the source of the Ca^{2+} providing feedback regulation of the $InsP_3$ receptor is provided extracellularly via Ca^{2+} influx across the plasma membrane. Rather than the detection of internal Ca^{2+} stores by the receptor or an associated protein, Ca^{2+} influx through the $InsP_3$ channel itself may modulate its gating throughout a large Ca^{2+} concentration range. Secondarily permeation is affected at high Ca^{2+} concentrations, blocking channel conductance, perhaps as a type of negative feedback loop. Ca^{2+} ions have been reported to inactivate the very channel they pass through (Yue, Backx, and Imredy, 1991), necessitating that the conducting ions that accumulate near the pore exert a negative feedback on Ca^{2+} flux as modeled by DeFelice (1993).

The long open modes observed in both the small- and large-conductance $InsP_3$-gated channels of lobster ORNs has never been reported for cerebellar $InsP_3$-gated channels, nor is the function of modal kinetic schemes in general well understood. Modal gating is inferred when a channel changes its kinetic behavior suddenly and maintains it for several seconds in the absence of solution changes or changes in membrane potential (Delcour, Lipscombe, and Tsien, 1992). Long open modes have been observed in a number of systems utilizing Ba^{2+} as a current carrier (Hess, Lansman, and Tsien, 1984; Nowycky, Fox, and Tsien, 1985; Pietrobon and Hess, 1990; Mazzanti, Galli, and Ferroni, 1992) to last for hundreds of milliseconds with only brief closures, much like Mode 1 or Mode 2 $InsP_3$-gated channels in our study. Three different modes of gating occur in N-type Ca^{2+} channels in bullfrog sympathetic neurons (Delcour et al., 1993), with low, medium, and high Pr_{open} comparable to the Pr_{open} of 0.15, 0.33, and 0.91-1.00 of the $InsP_3$-gated channels in our system. Spontaneous changes in the pattern of gating could influence the total contribution of the $InsP_3$-gated channels to Ca^{2+} or cation entry. For example a transition from normal Pr_{open} gating (0.33) to high Pr_{open} gating (0.91-1.00, Mode 1-2) could greatly increase the amount of current flow across the plasma membrane. Concurrently, a transition from normal Pr_{open} gating (0.33) to low Pr_{open} gating (0.15, Mode 3) could downregulate the total ion entry or current produced. As suggested by Delcour et al. (1993), a mode might be the expression of a modulatory state of a channel, superimposed on the set of conformational states occupied during normal gating. Because we observed the long open modes infrequently, in at most 10% of the channels recorded, one possibility is that the long openings could be attributed to spontaneous lapses in Ca^{2+} block of the conducting channel (see section above; DeFelice, 1993). Another possibility is that local ligand concentration, phosphorylation state, or GTP-protein binding could favor mode transition in the $InsP_3$-gated channels (Bean, 1989; Elmslie, Zhou, Jones, 1990; Yue, Herzig, and Marban, 1990).

Ionic Selectivity and Pharmacology

The failure of the mean reversal potential of the macroscopic odor-evoked inward current to coincide with the calculated equilibrium potential for any ion species, implies that the whole-cell excitatory current is a mixed current. A large percentage of the current (74-84%) may be mediated by an underlying Ca^{2+} channel, since it can be blocked by Co/Cd and RR. This is consistent with the pharmacological response of the underlying $InsP_3$-gated channels that are also both blocked by these probes. Discrepancy arises in the pharmacology of $InsP_3$-activated currents in olfactory tissue, so far reported in catfish (Restrepo et al., 1990), moth (Stengl, 1993), and lobster (this chapter). Insect ORNs are TTX insensitive, blocked by TEA, and insensitive to externally applied Ca^{2+} channel blockers (Stengl, 1993). This is clearly at odds with the heterogeneous TTX pharmacology and TEA insensitivity of the odor-evoked inward currents in lobster, the TTX blockability of the small-conductance $InsP_3$-gated channel, and the RR and Co/Cd sensitivity of both the large- and small-conductance $InsP_3$-gated channels. $InsP_3$-gated channels in catfish and lobster ORNs, are sensitive to Ca^{2+} channel blockers, yet $InsP_3$-evoked currents in catfish and moth ORNs are abolished by removal of external Ca^{2+} or Ca^{2+} chelation. The evidence that both the small- and large-conductance $InsP_3$-gated channel can be recorded in solutions where Ca^{2+} is the predominant ion and also in EGTA-chelated conditions, suggests that the current carried by each channel can also be carried by Na^+ in the absence of a Ca^{2+}-requiring mechanism.

In the lobster, ionic substitution demonstrates that half of the small-conductance $InsP_3$-gated channels are non-selective to cations, while the remaining half are intermediary between Na^+ and Ca^{2+}. Although based upon a more limited data sample, the large-conductance $InsP_3$ may be more selective for Ca^{2+} over other cations. In regards to Ca^{2+} permeability (not selectivity), the olfactory $InsP_3$-gated channels (Restrepo et al., 1990; Stengl, 1993; this chapter) are not unlike those that occur in the cerebellum (Maeda et al., 1991). Only the catfish olfactory $InsP_3$-gated channel is highly Ca^{2+} selective and shows more than one conductance state, being most like cerebellar IP_3-gated channels. In lobster, at least two different channels are gated by $InsP_3$ (Chapter 5), not a single channel with multiple conductive states as is true of $InsP_3$-gated channels in the cerebellum (Watras et al., 1991).

When one considers that the lobster $InsP_3$-gated channels are recognized by an antibody targeting a mammalian cerebellar $InsP_3$ receptor (Ca^{2+} channel)(Chapter 5), are sensitive to drugs targeting Ca^{2+} channels, and have either a non-selective cation, Na^+ and Ca^{2+} selective, or Ca^{2+} selective permeability, it is tempting to speculate that the $InsP_3$-gated channels in lobster represent evolutionary or developmental intermediaries between Na^+ and Ca^{2+} channels. Such a suggestion has been made for Na^+ currents in jellyfish that are blocked by pharmacological probes targeting Ca^{2+} channels (Anderson, 1987). Subsequent molecular sequencing confirmed the jellyfish Na^+ channel to be more closely homologous to Na^+ channels than Ca^{2+}, and revealed a different selectivity filter than that reported in higher animals (Anderson, Holman, and Greenberg, 1993).

Data support a subtle distinction between the two $InsP_3$-gated channels in lobster ORNs. Although separated by conductance, kinetics, and voltage dependence (Chapter 5), the channels cannot be distinguished based upon modulation by increased pH or Ca^{2+}, modal gating patterns, pharmacology, nor ionic selectivity. Although the latter properties fail to provide functional distinction between the two $InsP_3$-gated channels, they may help define the novel properties of olfactory, plasma membrane $InsP_3$-gated channels.

Modulation of output is typically shaped by the presence of more than one channel type in most neurons. The apparent redundancy of having more than one channel gated by the same second messenger, however, is unclear. Diversity in olfactory receptor neurons is classically attributed to differential expression of odor receptor proteins across a population of cells as well as to differential tuning of a single expressed receptor to different odor qualities. The excitatory transduction mechanism in lobster ORNs has the capacity to recruit at least 3 and possibly 4 types of second messenger-gated ion channels: a 30 pS $InsP_3$-gated non-selective cation channel, a 74 pS $InsP_3$-gated non-selective cation channel, a 193 pS IP_4-gated Ca^{2+} selective channel (Chapter 7), and possibly a 74 pS $InsP_3$-gated Ca^{2+}

selective channel. The distribution and type of second messenger-gated ion channel ultimately and perhaps equally contributes to ORN diversity.

CHAPTER 7
IP4-GATED ION CHANNELS IN NEURONS

Introduction

Stimulus-induced turnover of phosphoinositide is a major intracellular signalling system that mediates the action of neurotransmitters, hormones, and growth factors (Figure 7-1; Irvine, 1990; Neher, 1992a; Putney, 1992, Berridge, 1993). Inositol 1,3,4,5-tetrakisphosphate [$InsP_4$(1,3,4,5)], either alone (Higashida and Brown, 1986; Hill et al., 1988; Ely et al., 1990; Gawler et al., 1990; Parker and Ivorra, 1991; De Waard et al., 1992; Guse et al., 1992; Lückhoff and Clapham, 1993) or in combination (Irvine and Moor, 1986, 1987; Morris et al., 1987; Changya et al., 1989a, 1989b; DeLisle et al., 1992) with inositol 1,4,5-trisphosphate [$InsP_3$(1,4,5)], enhances Ca^{2+} entry into cells, sequesters or mobilizes Ca^{2+}, or triggers a change in membrane potential. Whether $InsP_4$(1,3,4,5) directly activates channels in neurons and the extent to which these receptors interact with $InsP_3$(1,4,5) receptors in excitable cells is unknown. We report that lobster olfactory receptor neurons (ORNs) express an $InsP_4$(1,3,4,5) receptor that is a

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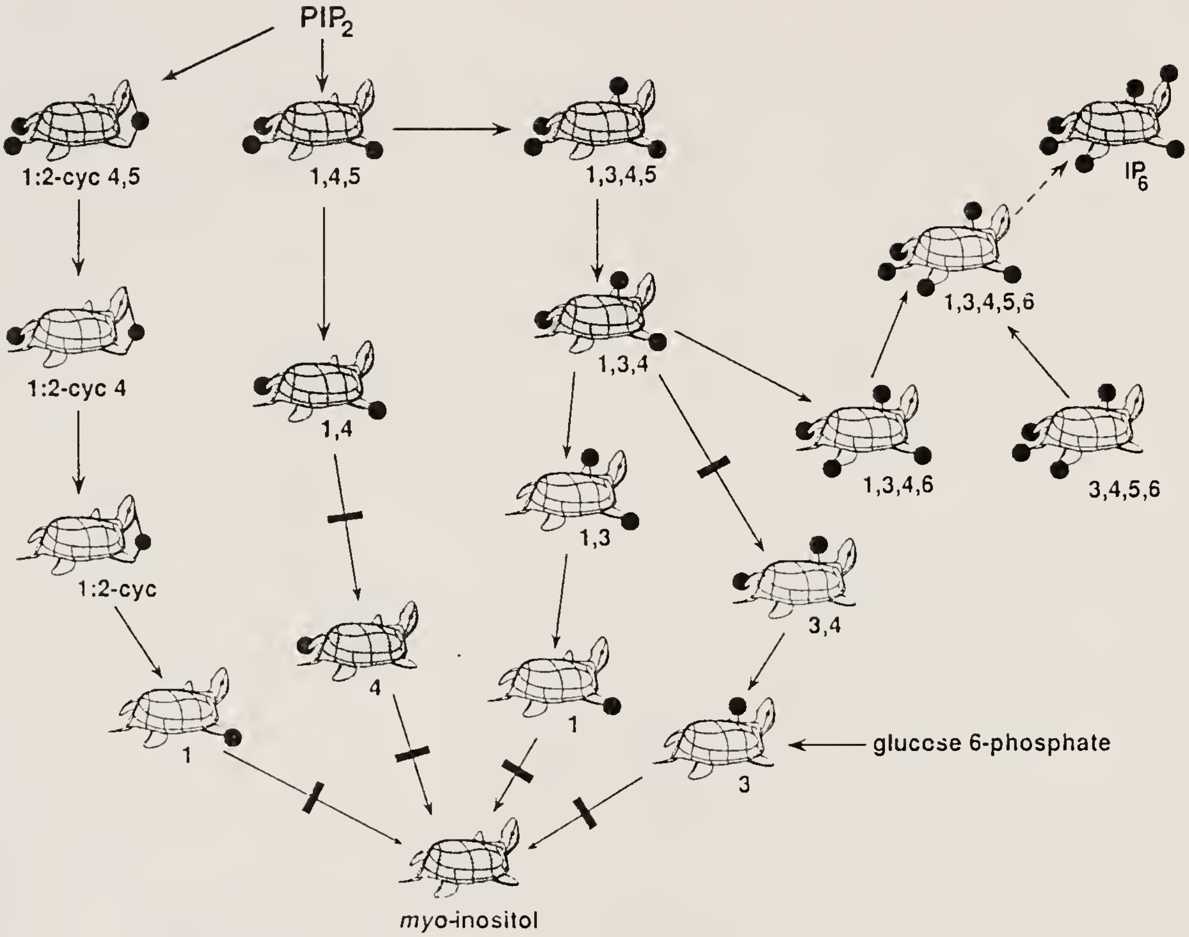
NOTE: This chapter will be submitted for publication in the journal Nature and conforms to its style requirements.

Figure 7-1. A non-threatening view of the inositol phospholipid metabolic pathway. The shell of the turtle represents the inositol ring, the solid circle the phosphate moiety, and the head & tail of the turtle, the chair conformation of the molecule. The shaded molecules are the inositol phosphates that were tested to determine whether they could evoke channel activity in inside-out cell-free patches of lobster ORN plasma membrane.

Turtles representing the inositol moiety were first presented by Dr. B. Agranoff and are used with his permission.


PIP$_2$
1:2-cyc 4,5
1,4,5
1,3,4,5
IP$_6$
1,3,4,5,6
1:2-cyc 4
1,4
1,3,4
1,3,4,6
3,4,5,6
1:2-cyc
1,3
3,4
4
1
1
3
glucose 6-phosphate
myo-inositol

functional channel differing in conductance, kinetics and voltage sensitivity from two previously reported plasma membrane $InsP_3(1,4,5)$-gated channels (Chapters 5-6). $InsP_4(1,3,4,5)$-gated channels interacted with the $InsP_3(1,4,5)$-gated channels by altering the Pr_{open} of the channels in what may be a novel mechanism for regulating Ca^{2+} entry. $InsP_4(1,3,4,5)$-gated channels may be considered one arm of a dualistic mechanism by which inositol phospholipids (IP) regulate olfactory signal detection in these neurons.

Results and Discussion

$InsP_4(1,3,4,5)$ (6.4×10^{-6} M) activated unitary currents in 25 of 42 cell-free patches of plasma membrane taken from cultured lobster ORN somata (Chapters 2-3; Figure 7-2). The average slope conductance of this channel (193 ± 13.0 pS, n=19) was 3-6 times that of two $InsP_3$-activated channels (30 pS, 74 pS) present in these neurons (Chapter 5). The average open probability (Pr_{open}) for the 193 pS channel at -60 mV was 0.11 ± 0.02 (n=14) (Figure 7-3A). The mean open time (t_o) for the channel was 5.03 ± 0.97 msec (n=11) (Figure 7-3B). The Pr_{open} of the channel was voltage dependent; the voltage function described a bell-shaped curve that decreased at hyperpolarizing and depolarizing extremes (Figure 7-4). Together with conductance, these gating properties distinguished the 193 pS channel from the two $InsP_3(1,4,5)$-gated channels, which have two open states

Figure 7-2 (A) Unitary currents activated by applying 6.4 x 10^{-6} M inositol 1,3,4,5-tetrakisphosphate [$InsP_4$(1,3,4,5)] to the inside face of a patch of membrane pulled from the soma of a lobster olfactory receptor neuron (ORN). The patch was voltage-clamped at various holding potentials. C= closed state, O_1 = one open channel, O_2 = two open channels. (B) X-Y plot of the amplitude of the unitary currents shown in A as a function of holding potential. Each data point represents the mean unitary current of at least 12s of open channel events determined based on a gaussian distribution fit of a point-by-point amplitude histogram at each holding potential. The data points were fit by locally weighted regression (continuous line). The mean conductance, determined from the slope of a linear regression at negative holding potentials for 19 channels, was 193 ± 13.0 pS.

METHODS. Distinct clusters of ORNs were dissected from the olfactory organs (lateral antennular filaments) of adult specimens of the Caribbean spiny lobster, *Panulirus argus*, enzymatically dissociated, and sustained in primary culture as described previously (Chapters 2-3). The cultured cells were viewed at 40X magnification with Hoffman optics for patch-clamp recording. Patch electrodes, fabricated from 1.8 mm O.D. borosilicate glass and fire polished to a tip diameter of approximately 1.0 μm (bubble number 4.8), produced seal resistances between 8 and 14 GΩ. Selected phospholipids were "spritzed" on the inner face of cell-free patches utilizing a previously described delivery system (Chapter 2). Unitary currents were recorded in symmetrical solutions, amplified, and processed as in an earlier study (Chapter 5). Briefly, signals were amplified with an integrating patch amplifier (Dagan 3900). The analog records were filtered at 2 kHz and recorded on videotape. On playback, the records were sampled every 100 μsec for processing on an IBM-compatible computer, using pCLAMP software (Axon Instruments). Patch solution (in mM): 30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 180 K-acetate, and 696 glucose, pH 7.0.

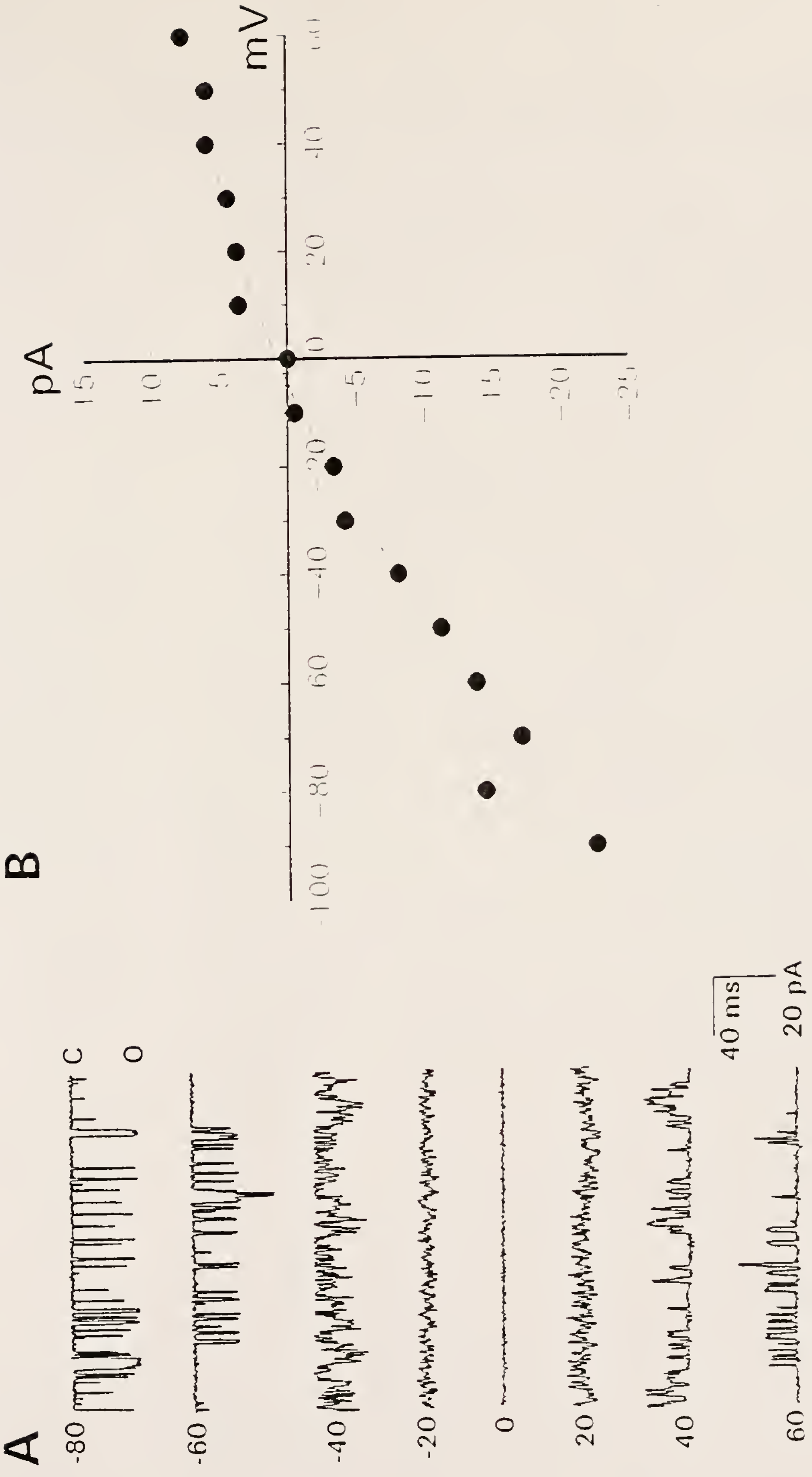

A
C
O
-80
-60
-40
-20
0
20
40
60
40 ms
20 pA
B
pA
mV
15
10
5
0
-5
-10
-15
-20
-25
-100
-80
60
-40
-20
20
40
60

Figure 7-3 (A) Histogram of the point-by-point amplitude of a digitized record containing three 193 pS channels activated by 6.4×10^{-6} M $\text{InsP}_4(1,3,4,5)$ in a cell-free patch from a lobster ORN. 36432 open channel events were fit by gaussian distributions (continuous line). C= closed state, O_1 = one open channel, O_2 = two open channels, O_3 = three open channels. The mean open probability (Pr_{open}), calculated from the area under the distributions, for 14 such ORNs at -60 mV was 0.11 ± 0.02. Inset shows a portion of the record used to generate the histogram. The density of the 193 pS channel was relatively high: patches containing 3 to 9 channels were not uncommon. Given an average surface area of 380.13 μm^2 for the soma of cultured ORNs (Chapter 3), the calculated average density of the 193 pS channel was 0.60 ± 10.80 μm^2. (B) Histogram of the open dwell time (t_o) of 2052 openings of a single 193 pS channel activated by 6.4×10^{-6} M $\text{InsP}_4(1,3,4,5)$ in a cell-free patch from a lobster ORN. The distribution was best fit by a single exponential (continuous line). Mean t_o = 5.03 ± 0.97 msec (n=11).

METHODS. To calculate the lower and upper channel density of the $\text{InsP}_4(1,3,4,5)$ channel the following were taken into consideration to calculate between 228 and 4105 channels per neuron: average surface area = 380.13 μm^2, percentage of patches containing the 193 pS channel = 60%, electrode tip diameter = 0.5 - 1.0 μm, and number of channels per patch = 1 - 9. For patches containing more than one channel the open probability was calculated as

$$Pr_{open} = \frac{0(P_0) + 1(P_1) + 2(P_2) + 3(P_3) + \ldots}{N}$$

where N = number of channels, P_0 = closed state probability, P_1 = probability of one channel open, P_2 = probability of two channels open, etc.

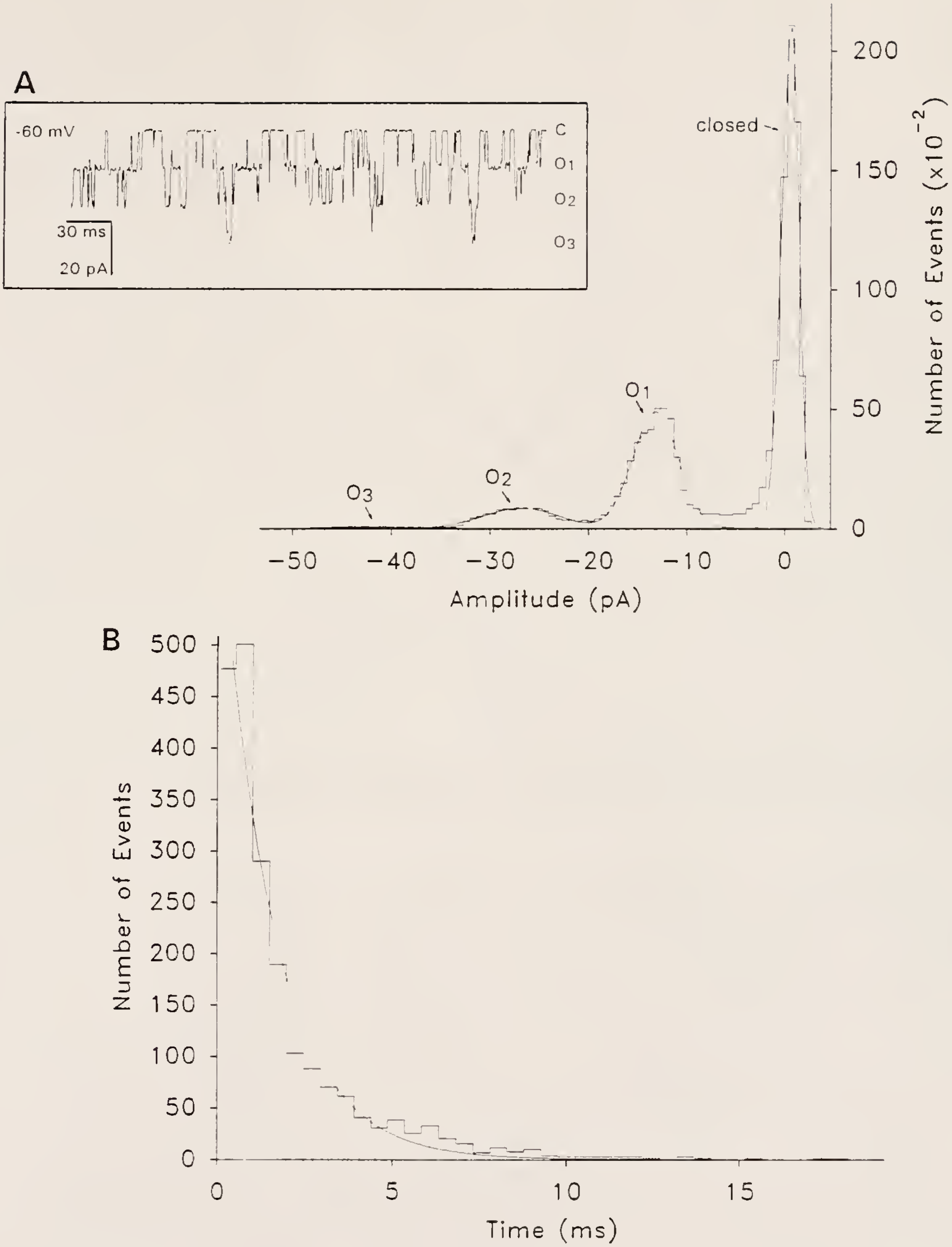
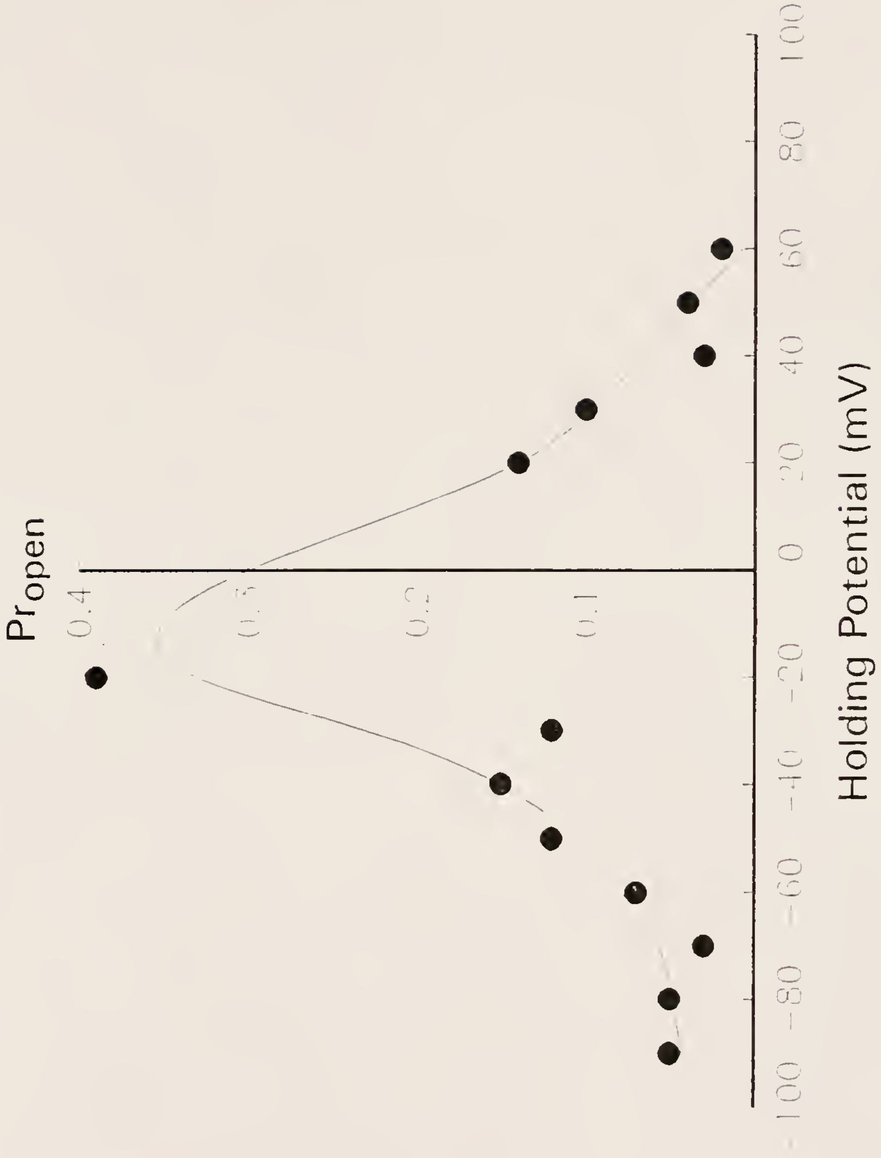

A
-60 mV
C
O1
O2
O3
30 ms
20 pA
closed
O1
O2
O3
200
150
100
50
0
Number of Events (x10^{-2})
−50
−40
−30
−20
−10
0
Amplitude (pA)
B
500
450
400
350
300
250
200
150
100
50
0
Number of Events
0
5
10
15
Time (ms)

Figure 7-4. Plot of the open probability (Pr_{open}) of a 193 pS channel activated by $InsP_4$(1,3,4,5) in a cell-free patch from a lobster ORN as a function of holding potential. The data were fit by a locally weighted regression (continuous line). Values at and bordering the reversal potential (-10 to +10 mV) could not provide a meaningful Pr_{open} calculation and were excluded from the fit.


Pr$_{open}$
0.4
0.3
0.2
0.1
-100
-80
-60
-40
-20
0
20
40
60
80
100
Holding Potential (mV)

and either decrease their Pr_{open} throughout the depolarizing range or are voltage independent (Chapter 5).

The 193 pS channel was selective for $InsP_4$(1,3,4,5) in 25 (86%) of 29 patches (Table 7-1). In the remaining four (14%) patches, $InsP_4$(1,3,4,5) activated unitary currents with average slope conductances similar to those of the $InsP_3$(1,4,5)-activated channels (29.9 ± 4.8 pS, n=3; 90.5 pS, n=1). The 30 and 74 pS channels were selective for $InsP_3$(1,4,5) in 104 (95%) of 110 patches. Of seven other metabolites in the inositol phospholipid pathway, $InsP_3$(1,4,5) (2.4×10^{-7} M) activated the 193 pS channel, in only 6 (5%) of 110 patches as did inositol hexaphosphate ($InsP_6$) (10^{-5} M) in one patch. None of the other metabolites gated channel activity. The inactivity of inositol 1,3,4-trisphosphate [$InsP_3$(1,3,4)] excluded the possibility that the 193 pS channel was activated by membrane associated 3-phosphatases secondarily generating $InsP_3$(1,3,4) from $InsP_4$(1,3,4,5) (Irvine, 1991).

As the solutions tested free of contaminating inositol phospholipid metabolites, we presume that the relatively low incidence at which $InsP_3$(1,4,5) activated the 193 pS channel and $InsP_4$(1,3,4,5) activated the 30 and 74 pS channels reflects low affinity cross receptor binding. Other inositol metabolites bind $InsP_4$(1,3,4,5) and $InsP_3$(1,4,5) receptors in mammalian brain only at high concentrations and with low affinity (Enyedi and Williams, 1988; Ferris et al.,

Table 7-1. Selectivity of various metabolites of the inositol phospholipid pathway for three different types of channels in cell-free patches of cultured lobster ORNs. $InsP_3(1,4,5)$ and $InsP_4(1,3,4,5)$ were commercial lots tested by NMR and hplc as 99 and 95% pure, respectively. Analysis did not detect contaminants as products of phospholipid metabolism.

Phospholipid	Concentration (M)	No. of Patches	No. of Patches Containing Channels	Mean Channel Conductance		
				30 pS	74 pS	193 pS
Inositol	10^{-3}	7	0	0	0	0
Inositol 1-monophosphate	10^{-5}	8	0	0	0	0
Inositol 1,2-cyclic monophosphate	10^{-5}	9	0	0	0	0
Inositol 1,4-biphosphate	3×10^{-4}	7	0	0	0	0
Inositol 1,3,4-trisphosphate	10^{-5}	9	0	0	0	0
Inositol 1,4,5-trisphosphate	2.4×10^{-7}	184	110	61	43	6
Inositol 1,3,4,5-tetrakisphosphate	6.4×10^{-6}	42	29	3	1	25
Inositol hexaphosphate	10^{-5}	13	2	0	1	1

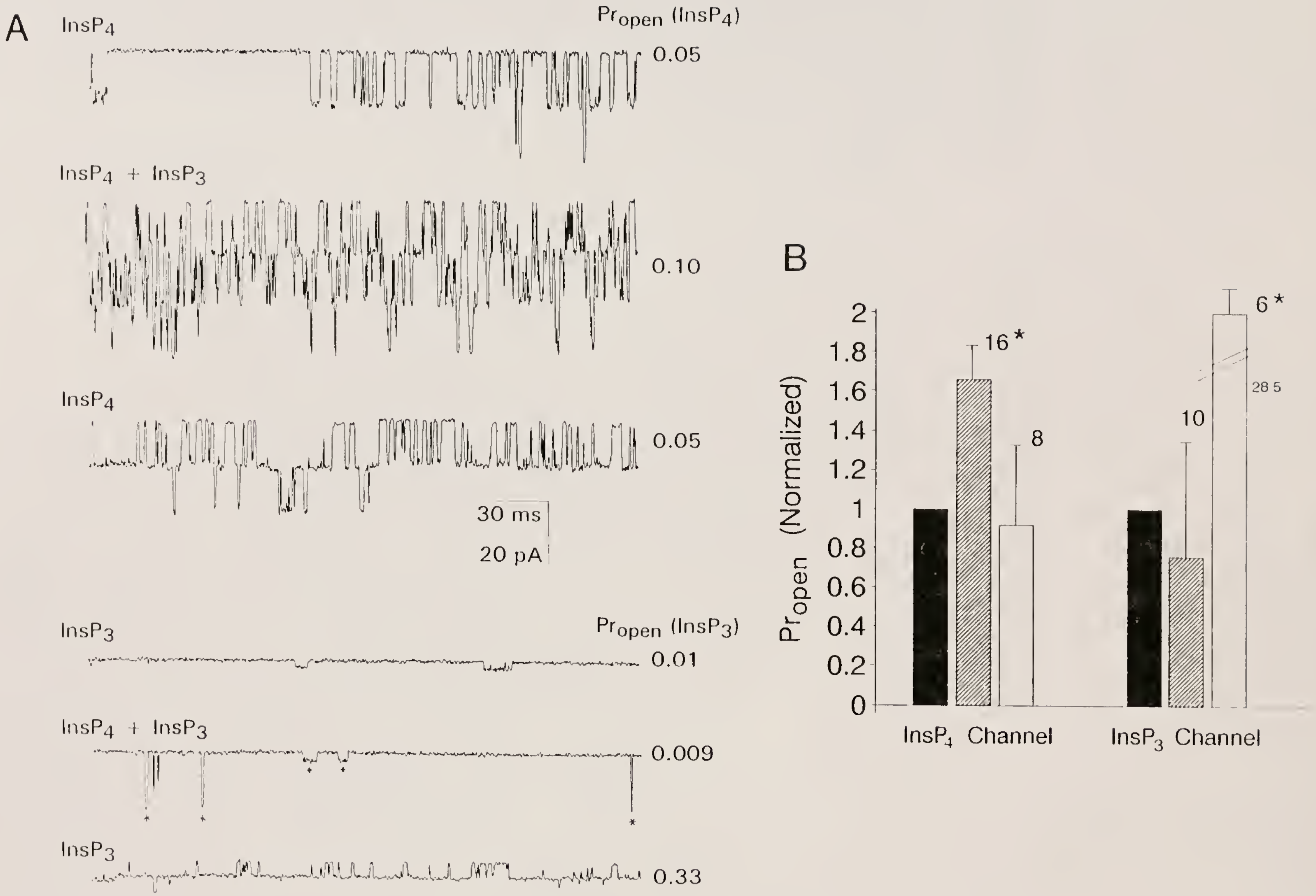
1989; Theibert et al., 1991, 1992). The apparent selectivity of the $InsP_3(1,4,5)$-gated channels in the lobster, is greater than that reported for the catfish olfactory $InsP_3(1,4,5)$ receptor, which binds $InsP_3(1,4,5)$ and $InsP_4(1,3,4,5)$ with equal affinity (Kalinoski et al., 1992).

The $InsP_4(1,3,4,5)$- and $InsP_3(1,4,5)$-gated channels interacted when they occurred in the same patch of membrane. Interaction altered the Pr_{open} but not the conductance of the channels (Figure 7-5A), suggesting an altered gating mechanism without interference of the pore region. In patches containing both types of channels, the Pr_{open} of the $InsP_4(1,3,4,5)$-activated channel significantly increased when the two ligands were co-presented, compared to that under $InsP_4(1,3,4,5)$ stimulation alone (n=16, paired t-test, Figure 7-5B). Interaction required that the $InsP_3(1,4,5)$-activated channel be gated and not simply present. The Pr_{open} of the $InsP_4(1,3,4,5)$-activated channel was not significantly different in patches that contained both IP-activated channels and patches that only contained $InsP_4(1,3,4,5)$-activated channels (n=8, Student's t-test, Figure 7-5B). The $InsP_4(1,3,4,5)$-activated channel, decreased the Pr_{open} of both $InsP_3(1,4,5)$-activated channels. In this direction, interaction only required the $InsP_4(1,3,4,5)$-activated channel be present, not gated. Co-presenting both ligands had no effect on the Pr_{open} of the

Figure 7-5. $InsP_3$- and $InsP_4$-gated channels interact to alter the open probability (Pr_{open}) but not the conductance state of the respective channels in cultured lobster ORNs.

A) Representative records of unitary currents activated by applying 6.4×10^{-6} M $InsP_4(1,3,4,5)$ and 2.4×10^{-7} M $InsP_3(1,4,5)$ to the inner face of cell-free patches of lobster ORNs. $InsP_4(1,3,4,5)$ (trace 1) and $InsP_3(1,4,5)$ (trace 4) activated the 193 pS and the 74 pS channel, respectively, in the same patch. Presenting both ligands simultaneously to the same patch as in traces 1 and 4 increased the Pr_{open} of the $InsP_4(1,3,4,5)$-activated channel (trace 2 vs trace 1), but not the $InsP_3(1,4,5)$-activated channel (trace 5 vs trace 4). Both $InsP_4(1,3,4,5)$- (*) and $InsP_3(1,4,5)$-(+) activated channel activity can be distinguished in trace 5. The Pr_{open} of the $InsP_4(1,3,4,5)$-activated channel in a patch that contained only this type of channel (trace 3) did not differ from that when both types of channels were present (trace 1). The Pr_{open} of the $InsP_3(1,4,5)$-activated channel, however, was greater in the absence of an $InsP_4$-activated channel (trace 6 vs trace 4). Pr_{open}, C, O_1, and O_2 as in Figure 7-2.

B) Bar graph comparing the normalized open probability (Pr_{open}) for all channels tested as in A. Solid bar = Pr_{open} for that channel when both channels were contained in the same patch in response to its own ligand. Striped bar = Pr_{open} for that channel when then co-presented with both ligands. * = Significant difference, $p \leq 0.05$; Arcsin transformation of percentage data followed by paired t-test. Open bar = Pr_{open} for that channel activated by its own ligand in patches known to only contain that type of channel. * = Significant difference, $p \leq 0.05$; Arcsin transformation of percentage data followed by Student's t-test.


A
InsP4
Pr_{open} (InsP4)
0.05
InsP4 + InsP3
0.10
InsP4
0.05
30 ms
20 pA
InsP3
Pr_{open} (InsP3)
0.01
InsP4 + InsP3
0.009
InsP3
0.33
B
$P_{r_{open}}$ (Normalized)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
16 *
8
10
6 *
28 5
InsP4 Channel
InsP3 Channel

$InsP_3$(1,4,5)-activated channels (n=10, paired t-test, Figure 7-5B), but the Pr_{open} of the $InsP_3$(1,4,5)-activated channel was significantly increased in patches that only contained $InsP_3$(1,4,5)-activated channels as opposed to patches that contained both IP-activated channels (n=6, Student's t-test, Figure 7-5B). Interaction between $InsP_4$- and $InsP_3$-activated receptors in other, non-excitable cells has been ascribed to a variety of mechanisms (Irvine, 1990, 1991). The ER $InsP_3$ receptor is reputedly locked into an inactive conformation by being bound to a plasma membrane $InsP_4$ receptor at the $InsP_3$ binding site. $InsP_4$ binding dissociates the coupled receptors to allow Ca^{2+} entry and free access of $InsP_3$ to its receptor. Such a model may not generalize to our system where both IP-activated channels are plasma membrane bound. Receptors in suitably close proximity in the same membrane might also be expected to block cytoplasmic binding sites; the presence of $InsP_4$-activated channels in lobster ORNs decreased the open channel activity of the $InsP_3$ receptor. Contrary to the model, however, the $InsP_3$-activated channel was stimulated in the absence of $InsP_4$ and in patches that did not contain $InsP_4$-activated channels.

We suspect that the 193 pS channel characterized in the soma functions in olfactory transduction. Cultured lobster ORN somata express transduction elements that are otherwise confined to the outer dendrite, the presumed site of odor transduction *in situ* (Chapters 2-6). In preliminary experiments (Ache et al., in press), a channel activated by $InsP_4(1,3,4,5)$, and two activated by $InsP_3(1,4,5)$, all with conductances within picosiemens of those reported in culture (Chapters 5 & 7), were recorded in the outer dendrite. Odors rapidly and transiently stimulate the production of $InsP_3(1,4,5)$ (Ache et al., in press), the metabolic precursor of $InsP_4(1,3,4,5)$, in the outer dendrite. Odor-stimulated production of $InsP_3(1,4,5)$ would increase Ca^{2+} influx through the $InsP_3(1,4,5)$-activated channels (Chapter 6), allowing activation of a Ca-dependent 3-kinase to produce $InsP_4(1,3,4,5)$ to gate the 193 pS channel. Such a large conductance channel could serve to enhance detection of the odor signal by rapidly increasing the onset of the receptor potential.

If, as in other systems, the $InsP_4(1,3,4,5)$-activated channel in lobster ORNs is calcium permeable, as preliminary experiments suggest (n=2, data not shown), then multiple channels would mediate Ca^{2+} entry into the cell. Dual receptors mediating Ca^{2+} flux have been implicated in controlling fertilization (Galione et al., 1993; Lee et al., 1993) and two Ca^{2+} releasing channels have been colocalized immunocytochemically in brain (Sharp et al., 1993). Having more than one Ca^{2+} mobilizing channel may be a fundamental property of excitable cells such as olfactory receptor neurons and interaction among the channels may provide fine control of neuronal output.

CHAPTER 8
SUMMARY

Sensory transduction pertains to all the mechanisms by which a specific type of stimulus energy is transformed into an electrical response by a specialized sensory cell: detection, amplification, encoding & discrimination, adaptation & termination, sensory channel gating, electrical response, and finally transmission to the brain (Shepherd, 1991). A variety of transductory elements - from putative G-protein coupled receptors to second-messenger gated ion channels - have been uncovered in the past four years, rapidly changing our view of olfactory signalling and detection. The data presented in my dissertation focused primarily on one specific stage of transduction, sensory channel gating (Chapters 5-7), and to a lesser extent on detection, discrimination (Chapter 3), and amplification (Chapter 4).

At the onset of my research, I developed appropriate *in vitro* conditions to sustain lobster olfactory receptor cells in primary cell culture (Chapter 2). Neurite outgrowth and cell viability were strongly affected by choice of adherent substratum, presence of serum, and length of animal captivity. Neither nerve growth factor, HEPES, nor preconditioned media from the target organ, the olfactory lobe, had any observable effect on either longevity or neurite outgrowth. The diversity of known growth factors (i.e. insulin, EGF, PDGF, etc.) in other systems has greatly expanded since my initial experiments and it would be worth while to pursue these as potential neurotrophic factors for lobster ORNs. Whether or not a required trophic factor or trace element could have sustained the neurons beyond the average 23 days remains unanswered. Nonetheless, a cell line was not required for successful physiological recording or, presumably, receptor protein expression. The defined culture conditions for these peripheral neurons can probably be modified to sustain other marine invertebrate neurons, including lobster CNS neurons, *in vitro*.

The finding that cultured ORNs maintained responsiveness to odors (Chapter 2) yet were morphologically more compact than their counterparts *in situ* lead to the prospect of using these dissociated cultured neurons to study the transduction elements that were normally confined to the relatively inaccessible outer dendrites. The cultured ORNs had to be surveyed for their degree of odor sensitivity and selectivity to discern whether they were an appropriate model (Chapter 3). The nature of the adequate stimuli, the degree of tuning (response spectra) of the cells, the threshold of sensitivity, the resting biophysical properties, and the dual polarity of the odor-evoked currents were all consistent with chemosensitivity in the cultured cells being olfactory, mimicking that of the cells *in situ*.

The ability of odors to evoke currents in cultured ORNs that lacked processes suggested that lobster ORNs could be induced *in vitro* to insert all elements of the transduction cascade in the soma, including those that normally may have been confined to processes. This greatly facilitated analysis of transduction in these cells as Chapters 4-7 attest. Most recently, inositol 1,4,5-trisphosphate- (IP_3) and inositol 1,3,4,5- (IP_4) gated ion channels were recorded in a vesiculated preparation of lobster outer dendrites, suggesting by localization that these second messenger-gated channels were transductory in nature (Ache and Hatt, in preparation). Riveting was the finding that these ion channels in the outer dendrite had estimated conductances and heparin & voltage sensitivity identical to those channels characterized in culture (Chapters 5-7); further substantiating the use of cultured neurons.

The finding that GTP-binding proteins linked odorant stimulation to the generation of both outward (inhibitory) and inward (excitatory) currents in lobster ORNs (Chapter 4) was not unexpected given the rich evidence for G protein mediation of olfactory signalling in other species, and the discovery of a multigene family of putative olfactory receptors coupled to G proteins. What G protein subtype mediated these transductory currents was unclear, especially as other olfactory related G proteins demonstrated ribosylation and the lobster ORNs could not be functionally blocked by the bacterial toxins. It must be concluded that at least one of the transductory G proteins is resistant to ribosylation as even injection of an active subunit does not alter odor-evoked current magnitude. The lobster ORNs probably contain a G protein of the α_q family, all of which are PTX insensitive. A second G protein, more similar to G_o of the α_i family, is likely coupled to excitatory odor transduction in lobster ORNs. One or the other or both G proteins, coupled to one or several cell-surface receptors, may create a heterogeneous expression pattern across a population of ORNs to give each receptor its own blueprint of excitatory receptor cell output. As the odor-evoked outward (inhibitory) currents are clearly G protein dependent, and the nature of this protein subtype remains undetermined, I could easily envision another, yet defined, class of G proteins mediating this transduction cascade. The α_q family was only discovered three years ago and its enzymatic effector clearly defined two years ago. I feel that this multigene family will be a target of intensive research as it is so highly conserved across eukaryotes. Which G protein subtypes functionally couple to what enzyme or ion channel effector is an unexplored avenue in olfactory transduction.

The finding outlined in Chapter 5 that IP_3 directly gates an ion channel in the plasma membrane was the first evidence for such a channel in neurons. The IP_3-activated channels had kinetic properties of odor-activated channels in the ORNs and pharmacological properties of intracellular IP_3-activated channels in other systems. An antibody directed against an intracellular, cerebellar IP_3 receptor recognized a protein in the lobster ORNs of similar molecular weight to the mammalian receptor. Interestingly, the antibody selectively increased the magnitude of odor-evoked inward currents and the open probability of IP_3-activated unitary currents. The ability to insert a patch of membrane containing an IP_3-gated channel into a recipient cell, which could then be stimulated by an odorant to evoke channel activity, provided further evidence for IP_3 as an olfactory second messenger mediating excitation in lobster ORNs.

The discovery that two channels - differing in conductance, voltage dependence, and dwell-time kinetics - were gated by IP_3 (Chapter 5) lead to the investigation of the channels' intricate gating properties, ionic selectivity, pharmacology, and ligand specificity (Chapters 6-7). Although these distinctions did not provide strong evidence that the channels were functionally dissimilar to each other, they provided a body of knowledge about the novel biophysical properties of IP_3-gated channels in olfactory neurons, in comparison to those located intracellularly, as in the cerebellum. The finding that elevated pH or $[Ca^{2+}]_i$ increased the open probability, whereas the latter simultaneously decreased the conductance of both channels, implied that modulation of channel gating could affect opening and closing of the channel in addition to ion permeation. Both IP_3-gated channels displayed spontaneous transitions in gating kinetics across three distinct patterns or modes. While the presence of mode behavior is not well understood in channel biology, one could envision these long open modes the result of many molecular mechanisms, from transient lapses in open channel block, to changes in phosphorylation state of the channel protein.

Both the small- and large-conductance IP_3-gated channel mimicked the pharmacology of the macroscopic odor-evoked inward current. Although each were reversibly blocked by typical calcium channel blockers, the experiments to measure the channel reversal potential using ionically substituted conditions were inconclusive. Further permeation experiments are required to determine the extent to which these channels are Ca^{2+} selective.

What can be inferred from the current reversal data of Chapter 6, is that the excitatory current is clearly a mixed current, most probably comprised of several channel species. As direct odorant gating of receptors does not appear to be a likely transduction mechanism (unpublished data), it would benefit to use the cultured neurons for the investigation of other second messengers mediating the excitatory current, such as Ca^{2+}, G-protein gated ion channels, nitric oxide, other inositol phospholipid metabolites, or members of the eicosanoid pathway.

Finally with the knowledge that other phospholipids are suspected to serve as intracellular signals, a survey of IP_3 metabolites (Chapter 7) revealed that IP_4 directly activated an ion channel in the plasma membrane of the ORNs that differed in conductance, kinetics, density, and voltage sensitivity from the IP_3-gated channels. Most intriguing, I found that the IP_4-activated channel mutually interacted with the IP_3-activated channels to alter the open probability but not the conductance of the channel. IP_4-activated channels appear to be one arm of a dualistic, interactive mechanism by which inositol phospholipids regulate signal detection in these neurons.

My results suggest that our current model of olfactory transduction in lobster ORNs (Figure 8-1) is overly simplistic and will need to account for additional putative second messengers (e.g. IP_4), modulatory factors (e.g. Ca^{2+} or phosphorylation), or even cross-talk (regulatory interaction between cascades). Most importantly, the model must reflect potential alternative ways in which the transduction elements can be packaged within single cells.

Figure 8-1. Model of the dual, opposing second messenger cascades hypothesized to mediate olfactory transduction in lobster ORNs. Some odor molecules (triangle) bind to receptor proteins linked to an as yet undefined G-protein. This G-protein mediates adenylate cyclase conversion of ATP to cAMP, which directly gates a potassium channel to hyperpolarize the cell. Other odor molecules (square) bind to receptor proteins linked to a G_o- and/or G_q-like G-protein. These G-proteins mediate phospholipase C conversion of PIP_2 to IP_3, which directly gates a non-selective cation channel and possibly a Ca^{2+}-dependent channel to depolarize the cell. The output of the cell to the CNS depends on the net level of depolarization resulting from co-activation of the two transduction cascades.

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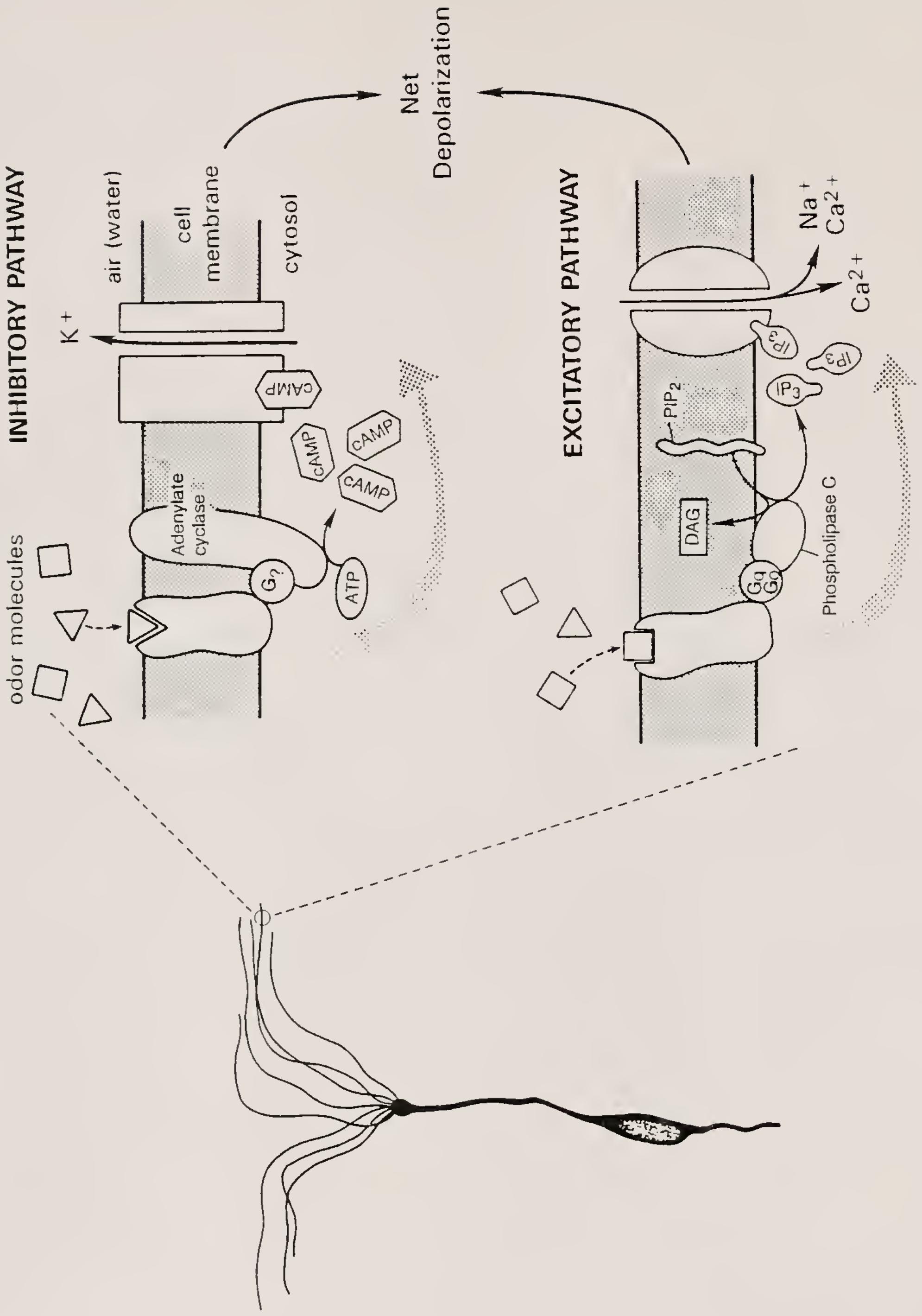
INHIBITORY PATHWAY
odor molecules
K +
air (water)
cell membrane
cytosol
Adenylate cyclase
G?
ATP
cAMP
cAMP
cAMP
cAMP
Net Depolarization
EXCITATORY PATHWAY
Na +
Ca2 +
Ca2 +
PIP2
IP3
IP3
IP3
DAG
Phospholipase C
Gq
Go

Distribution of opposing cascades (excitatory/inhibitory) and construction of cascades with different G proteins and second messenger-gated ion channel types has the potential to create diversity in receptor cell output. For example, a G_o-like protein coupled to IP_3 production, which secondarily gates a large conductance channel in a cell lacking an inhibitory cascade would produce a different signal than a cell containing G_q-like and G_o-like proteins simultaneously coupled to IP_3 production, which gates a small conductance channel in a cell that also contains the inhibitory cascade.

An emerging theory in the chemoreceptive field--"One receptor protein, one olfactory receptor cell"--is dominating meeting symposia and shaping predictive models in the literature (e.g. Lancet et al., 1993). In the absence of functional evidence, this theory remains just that. Electrophysiological and biochemical cross-receptor binding studies in the lobster and catfish are not consistent with such an exclusion model, although at this time, they are unable to rule it out entirely (Caprio and Byrd, 1984; Bruch and Rulli, 1988; Caprio et al., 1989; McClintock et al., 1989b, Michel et al., 1991, 1992b; Fadool et al., 1993). So the BIG question for lobster chemoreception remains, whether or not a single ORN can bind both excitatory and inhibitory odorant compounds to a single cell-surface receptor type that is linked to two or three different G proteins subtypes, which individually mediate at least the activation of effector enzymes in the production of second messenger molecules, which in turn clearly gate more than one type of ion channel. The ORN could finely tune its transmission to the brain by utilizing machinery from both transduction cascades inside a single cell (Ache et al., in press) but it remains to be understood whether this is accomplished with a single class of olfactory receptor proteins, or whether ORNs express multiple receptor proteins coupled individually to each cascade (Figure 8-1). Secondarily, it is not understood whether all cells express both transduction cascades, or only a subset possess multiple mechanisms.

Independent of receptor protein expression or cascade distribution across cells, lobster olfactory transduction clearly utilizes more than one pathway. My dissertation has not changed this proposition (as hypothesized in Chapter 1), but rather has provided details of the mechanisms. It has instigated a different perspective of olfactory signalling: the detection of odor qualities by an ORN is not only dependent upon the type and tuning of its olfactory receptor protein, but the type, distribution, modulation, and interaction of its second messenger-gated ion channels.

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BIOGRAPHICAL SKETCH

Debra Ann Fadool was born to Elizabeth Lynn Lewis and Robert Servetus Frey on 15 November 1962 in Highland Park, Illinois. When she was a year old, she and her family, including her sister Elizabeth Lynn, moved to Cincinnati Ohio, where she resided and attended grade school at Kilgore Elementary. When she was six, her father, her sister, and she moved to Louisville, Kentucky, where she completed grade school at Norton Elementary and Belknap Elementary. She attended middle school at Highland Jr. High School. She attended high school at Atherton High and Louisville Central, graduating in 1981. It was during these years that she became disciplined in athletics, competing nationally in synchronized swimming and qualifying for the All State cross country team. It was also during this period that she acquired a self-taught interest in science, which developed out of boredom for something constructive in a school system which was undergoing long awaited desegregation. She directed her high school science education by participating in state and regional math and science fairs, which gave her the incentive to pursue biology at Albion College, Michigan. There she fostered her childhood interest in teaching through the example of several excellent professors, and a love of marine biology through an off-campus semester in the U.S.V.I., St. Croix. She graduated magna cum laude from Albion College--MVP and all MIAA, NCAAIII cross country & track; biology and english majors, May of 1985. She taught at Newfoundland Harbor Marine Institute, Big Pine Key, Florida, the summer before going to Kingston, Rhode Island, where she earned a Master of Science jointly under the supervision of Dr. Phyllis Brown, an analytical chemist, and Gabriel Kass-Simon, a physiologist. She returned to Louisville and married James Michael Fadool at St. Paul's United Methodist Church. They resided in East Lansing, Michigan, where she completed additional classwork, taught biology lectures, and was a research associate for Michigan State University. She had her first son, James Calvin, the summer of 1989, shortly afterwhich the family moved to St. Augustine, Florida, to allow her to commence her doctorate research at the Whitney Laboratory under the direction of Barry Ache, a chemosensory physiologist. She studied neurobiology at Marine Biological Laboratory, Woods Hole, Massachusetts, the summer of 1991. She gave birth to her second son the spring of 1993, traveled to Sapporo Japan to participate in an international smell and taste meeting, and returned to St. Augustine to complete her requirements for the degree of Doctor of Philosophy in zoology through the University of Florida, December 1993. She looks forward to studying as a postdoctoral scientist under the direction of

Irwin Levitan, a membrane biophysicist at Brandeis University, Waltham, Massachusetts, while her husband studies under the direction of John Dowling, a developmental biologist at Harvard University, Boston, Massachusetts.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Barry W. Ache, Chairman
Professor of Zoology
and Neuroscience

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Peter A.V. Anderson
Professor of Neuroscience

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Barbara-Anne Battelle
Associate Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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William E.S. Carr
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michele G. Wheatly

Michele Wheatly
Associate Professor of Zoology

This dissertation was submitted to the Graduate Faculty of the Department of Zoology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1993

Dean, Graduate School

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